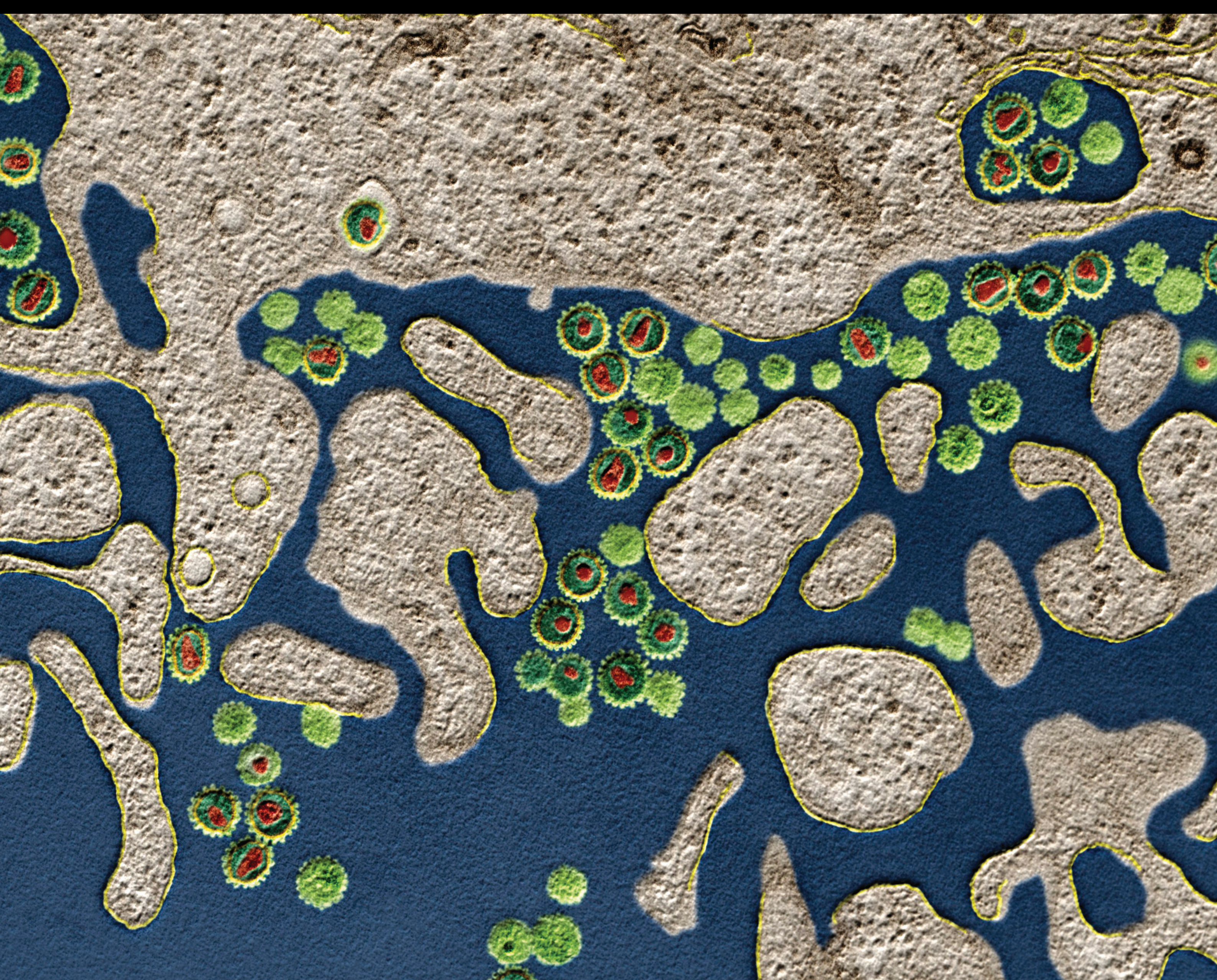
Journal of Immunology Research

Xenotransplantation: The Way Beyond and Ahead toward Clinical Application

Lead Guest Editor: Laura Iop
Guest Editors: Emanuele Cozzi and Vered Padler-Karavani



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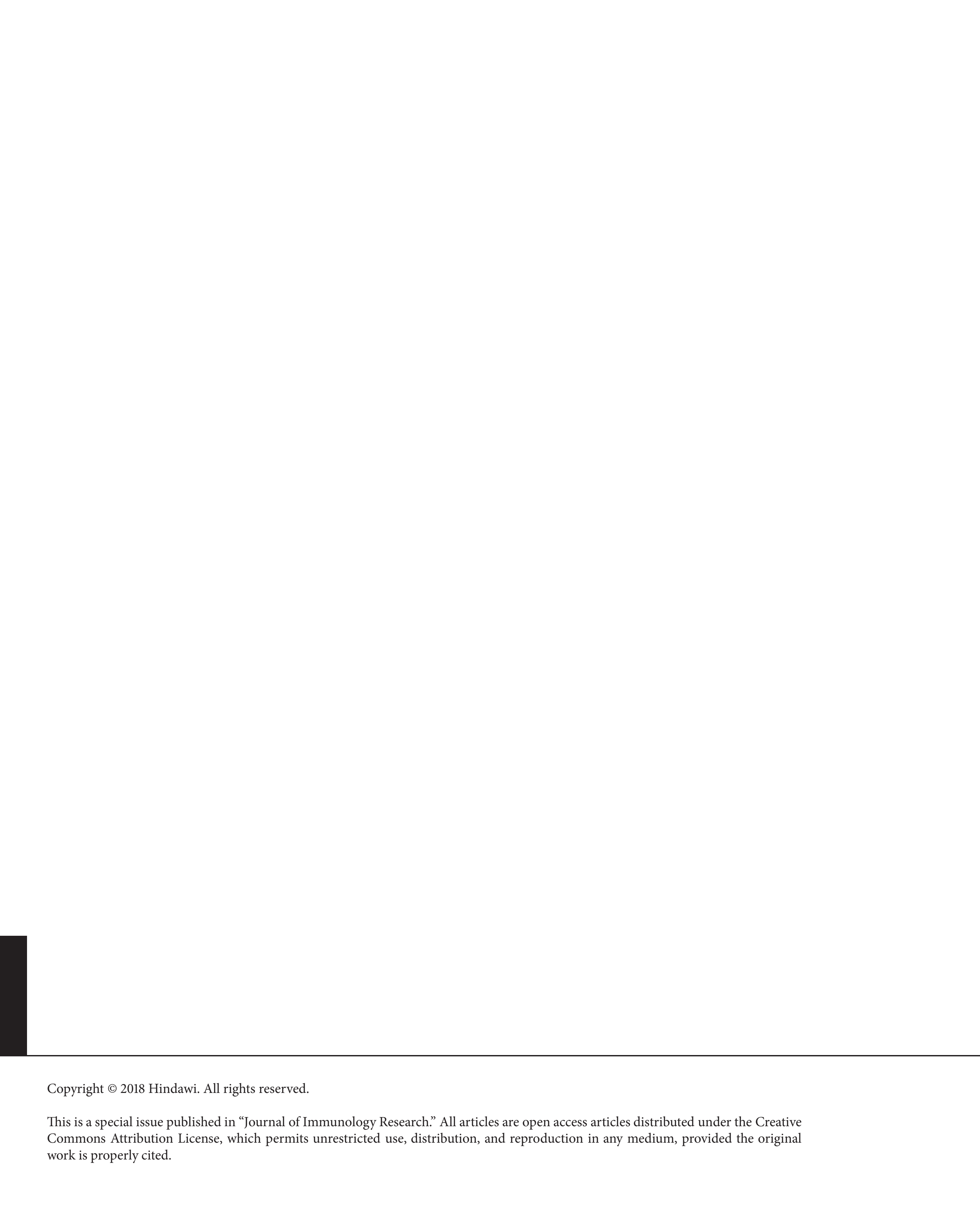

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Editorial

Xenotransplantation: The Way beyond and Ahead toward Clinical Application

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Instigated at the beginning of the 20th century, modern transplantation has triggered an unprecedented era for medical therapeutics. Indeed, the feasibility to successfully transplant cells, tissues, or organs derived from an allogeneic donor has opened the way to novel approaches to rescue and prolong the life of patients with end-stage organ failure. However nowadays, due to an increasing gap between the demand for organs and the limited number of human donors, allogeneic transplantation is, unfortunately, a therapeutic option that is not always available to those in need.

In the dramatic scenario of human donor shortage and increasing transplantation waiting lists, transplanting organs from other species such as the pig into man, a condition known as xenotransplantation, might represent an appealing therapeutic solution for a larger population of patients affected by organ failure. Despite the well-established similarities with regards to some aspects of anatomy and physiology between pigs and humans, transplanting pig tissues and organs into man is known to elicit an aggressive immune reaction that commonly results in premature failure of the graft.

Breakthroughs in the understanding of the mechanisms underlying xenograft rejection, that include advancements in glycoimmunology, proteomics, and molecular signaling, have enabled the development of novel modalities to prevent or delay the development of antixenograft immune responses. In fact, innovative strategies have been devised to modulate the recipient's immune response to xenografts, to reduce the immunogenic power of transplanted cells, tissues, and organs, or to create a physical barrier between the human blood and the xenograft. However, the route to the clinical application of xenotransplantation still presents many hurdles that will need to be overcome before reaching its ambitious therapeutic goal.

In this special issue, we aim to offer an overview of the state of the art of xenotransplantation with reference to the continuous innovation and potential issues in this challenging medical field. In particular, G. P. Yung and colleagues review the role of human natural killer (NK) cells in the pathophysiological mechanisms leading to xenograft rejection (in *The Role of NK Cells in Pig-to-Human Xenotransplantation*). In this context, the authors accurately describe specific molecular strategies to overcome the human NK response directed against porcine endothelial cells. As previously evidenced for NK immune responses, costimulatory molecules represent a key target of intervention to optimize immunomodulation in xenotransplantation. In the review by K. P. Samy et al. (*in The Role of Costimulation Blockade in Solid Organ and Islet Xenotransplantation*), the central role of such approaches is illustrated in enabling xenotransplantation of solid organs, as kidney, heart, and liver, as well as of islets. Specifically, advancements in terms of survival achieved in preclinical models with the introduction of monoclonal antibodies against CD154, CD40, and CTLA-4 are discussed. E. Montanari et al. cover another strategy that might possibly improve xenograft durability. This approach

is represented by the protection offered to hepatocytes by cotransplantation with multipotent mesenchymal stem cells (MSCs) (in *Beneficial Effects of Human Mesenchymal Stromal Cells on Porcine Hepatocyte Viability and Albumin Secretion*). In fact, alginate-encapsulated hepatocytes demonstrated higher viability and albumin secretion upon coculture with MSCs thanks to the paracrine effect of their secreted cytokines CCL2, CXCL12, and macrophage migration inhibitory factor (MIF). Such original results are of great interest for the success of cell transplantation in the setting of acute liver failure and once again, confirm the powerful immunomodulatory and protective abilities of MSCs when applied as ancillary therapies or alone.

Besides immune response, other pitfalls in the clinical application of xenotransplantation could be represented by inappropriate organ function and growth adaptation, also developed in the long term. J. A. Shah and colleagues analyze the possible causes of post-transplant proteinuria in preclinical renal xenotransplantation and summarize the preventive strategies that are currently being tested/applied (in *Potential Pathways behind Proteinuria as well as Factors Related to Growth Discrepancies following Pig-to-Kidney Xenotransplantation*). In addition, they highlight the importance of organ size match since discrepancies may ultimately be a cause of xenograft failure. Lastly, in their review, C. G. A. McGregor and G. W. Byrne examine the current barriers hindering access of heart xenotransplantation in the clinical arena (in *Porcine to Human Heart Transplantation: Is Clinical Application Now Appropriate?*). Following an accurate description of the technological progress that has enabled the current preclinical results in nonhuman models, the remaining key preclinical studies indispensable to ensure the efficacy and safety of clinical cardiac xenotransplantation are discussed.

In this continuous cross talk between fundamental and applied research, progress is very encouraging and clinical translation of novel therapeutic solutions based on the use of animal-derived cells, tissue, or organs appears increasingly closer.

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Review Article

Remaining Physiological Barriers in Porcine Kidney Xenotransplantation: Potential Pathways behind Proteinuria as well as Factors Related to Growth Discrepancies following Pig-to-Kidney Xenotransplantation

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Considerable shortages in the supply of available organs continue to plague the field of solid organ transplantation. Despite changes in allocation, as well as the utilization of extended criteria and living donors, the number of patients waiting for organs continues to grow at an alarming pace. Xenotransplantation, cross-species solid organ transplantation, offers one potential solution to this dilemma. Previous extensive research dedicated to this field has allowed for resolution of xenograft failure due to acute rejection, leaving new areas of unresolved challenges as barriers to success in large animal models. Specific to kidney xenotransplantation, recent data seems to indicate that graft compromise can occur due to discrepancies in growth between breeds of donors and significant proteinuria leading to nephrotic syndrome in the recipient. Given these potential limitations, herein, we review potential pathways behind proteinuria, as well as potential causative factors related to growth discrepancies. Control of both of these has the potential to allow xenotransplantation to become clinically applicable in an effort to resolve this organ shortage crisis.

1. Introduction

Xenotransplantation remains a promising avenue to address widespread shortages of available organs for transplantation. As a result of extensive previous research, largely in part due to recent advances in genetic engineering, xenografts suitable for human transplantation are on the verge of becoming a clinical reality. The first major breakthrough in the field of xenotransplantation came as a result of the creation of alpha-1,3-galactosyltransferase knockout (GalT-KO) pig donors which were successful in preventing the development of hyperacute xenograft rejection (HAR) [1–3]. More recently, the creation of multitransgenic (Tg) swine donors has allowed investigators to successfully experiment varying immunosuppression regimens consisting of a combination of costimulation blockade, mycophenolate mofetil, and T/B-cell depletion, the effects of which in a cardiac model have allowed for survival of heterotopic hearts in baboons for >2 years [4] and >6 months in a life-supporting kidney model [5, 6]. Despite the improvement in survival from days to months, additional barriers due to antigenic and physiologic differences in cross-species transplantation continue to remain a challenge [7–9].

Specific to challenges limiting xenogeneic kidney transplantation (XKTx), we hypothesize three limiting obstacles: the first is significant posttransplant proteinuria, the second appears to be related to organ growth disparities following xenotransplantation, and the third is the level of immunosuppression needed to control xenogeneic innate and acquired immune responses in an effort to prolong xenograft survival. This review is based predominantly on data from our own studies which focuses on the first two obstacles by reviewing potential pathways behind proteinuria as well as factors related to growth discrepancies. Control of both,

along with an adequate control of the xenogeneic immune response, has the potential to allow xenotransplantation to become a clinically applicable solution for addressing the organ shortage crisis.

2. Proteinuria and Nephrotic Syndrome Complicating Xenotransplantation

2.1. Proteinuria following Pig Kidney Grafts in Nonhuman Primates. Significant proteinuria has been previously reported [9–12] following α-1,2-fucosyltransferase pig-to-cynomolgus, as well as following GalT-KO and GalT-KO/hCD39/CD55/CD59 pig-to-baboon xenotransplantation [9–12]. Often, the proteinuria is in the nephrotic range and is associated with vascular thrombosis and infections that can limit the survival of the recipient. Histologically, the glomeruli appear normal or show mild mesangial expansion [12]. Although antibody-mediated rejection could lead to proteinuria [13], we have found that nephrotic syndrome can occur in the absence of any elicited antibody development or complement activation (i.e., antibody-mediated rejection) after pig-to-baboon xenotransplant [9, 12].

While nephrotic syndrome is a common complication following xenotransplantation, investigators at Emory have published data demonstrating low preformed natural antibodies and >133-day survival with minimal proteinuria and absence of serum hypoalbuminemia in rhesus monkeys that have received GalT-KO/hDAF (human decay-accelerating factor) kidneys [5]. Despite these encouraging findings, Pintore et al. continue to report challenges that occur with posttransplant proteinuria and the presence of low molecular weight proteins that are consistently found in recipient urine samples after kidney xenotransplantation in hDAF or multi-Tg GalT-KO pig-to-cynomologus monkey models [10, 11]. In addition to these findings, our lab has extensive experience with hDAF/GalT-KO pig-to-cynomolgus monkey XKTx using an anti-CD40L-based regimen without vascularized thymic grafts, and these recipients commonly developed proteinuria similar to that observed in baboon recipients of GalT-KO pig kidneys (Yamada et al., manuscript in preparation). Given the discrepancy in these results, it is not yet concluded whether specific recipient strains are involved in the absence of proteinuria that is observed in the GalT-KO/hDAF rhesus macaque model [5]. Efforts to resolve proteinuria remain a major focus for the success of XKTx.

2.1.1. Potential Causes for Posttransplant Proteinuria. Our laboratory has previously published data demonstrating that the cotransplantation of vascularized thymic grafts with kidneys from the same GalT-KO donor results in prolonged kidney survival in a life-supporting pig-to-baboon model [3, 12]. Recipient baboons in this study demonstrated in vitro evidence of donor-specific tolerance with the development of early baboon thymopoiesis in vascularized pig thymic grafts, suggesting that the recipients were on a path towards the induction of tolerance. The majority of the recipients however developed significant proteinuria as early as postoperative day (POD) 2, despite relatively normal appearing glomeruli and normal renal function [9, 12]. Based upon these results, it was concluded that the development of proteinuria is not initiated by T-cells or as a result of antibody-mediated rejection. Additionally, histologic examination revealed findings that were remarkably similar to the nephrotic condition known as minimal change disease (MCD) that is common in pediatric populations [14, 15]. Furthermore, the development nephrotic syndrome leads to significant anasarca and increases the risk of developing infections, cortical damage, and graft thrombosis due to microangiopathy. It deserves to be mentioned that although microangiopathy and infections are quite frequent complications following xenotransplantation [12, 16], the fact that these complications can result from nephrotic syndrome alone provides additional rationale to identify and treat the causative mechanism(s) [17].

2.1.2. Approaches to Preventing Proteinuria. A recent study reported that the loss of sphingomyelin phosphodiesterase acid-like 3b (SMPDL-3b) in allogeneic human kidney grafts was related to the development of posttransplant proteinuria in patients with focal segmental glomerulosclerosis (FSGS) [18]. In our laboratory, studies of the pig-to-baboon XKTx model have shown that the administration of rituximab in the perioperative period appears to protect SMPDL-3b/sphingomyelinase activity on porcine podocytes, which in turn delays the development of proteinuria [9]. Given that podocytes are one of the primary cells within the glomerulus, we developed a novel technique to study postxenotransplantation proteinuria using pig podocyte cultures followed by histologic confirmation by staining with antinephrin and antipodocin antibodies. As a result of this porcine podocyte culture, our lab was able to discover two critical findings: (i) SMPDL-3b/sphingomyelinase expression on porcine podocytes plays an essential role in initiating proteinuria and (ii) rituximab (anti-CD20 antibody) binds to porcine SMPDL-3b in the glomeruli of the kidney xenografts, thereby preventing damage from circulating baboon preformed antipig natural antibodies or antiporcine soluble factors.

Given our findings from in vitro studies, rituximab was administered to six baboons in the peritransplant period (treatment group) in order to test its effect in vivo, and the treatment was compared with eighteen baboons that underwent GalT-KO thymokidney transplantation without rituximab administration in the peritransplant period (control group). The onset of 2^{+} proteinuria posttransplant was markedly delayed in the treatment group when compared to the control group. Most of the baboons in the control group developed $>2^{+}$ proteinuria within 2 days following transplantation, as compared to the treatment group where the development of 2^{+} proteinuria occurred $>12.50 \pm 5.54$ days posttransplant. To our knowledge, these findings demonstrated for the first time the ability of rituximab to prevent pig podocyte disruption in an SMPDL-3b-dependent manner with subsequent delay in the development of proteinuria following xenogeneic GalT-KO kidney transplantation in nonhuman primates (NHPs) [9]. However, since this effect lasted only two to three weeks, additional treatment strategies are necessary.

2.1.3. The Role of CD80 Upregulation and CTLA4-Ig to Prevent Proteinuria. A significant breakthrough in understanding the pathogenesis of proteinuria occurred with the discovery that podocytes have antigen-presenting functions [19] and can express the dendritic cell receptor CD80 (also known as B7.1) [20, 21]. Recently, urinary levels of CD80 were reported to be extremely high in a patient who developed minimal change-like nephrotic syndrome following allogeneic stem cell transplantation [22]. Moreover, MCD, the most common cause of proteinuria and nephrotic syndrome in children, has been associated with high levels of CD80 in the urine as well as with CD80 expression in glomerular podocytes in renal biopsies [23, 24]. Children with relapsing MCD have been observed to express CD80 on their podocytes (as seen on renal biopsy) and excrete CD80 in their urine, both of which resolve when they enter remission [23, 24]. Serum from relapsing MCD patients has been shown to induce CD80 expression in cultured human podocytes. Additionally, toll-like receptor ligands 3 (polyIC) and 4 (endotoxin (LPS)) in mice induce glomerular CD80 expression and urinary CD80 excretion and cause transient proteinuria [21, 25–27]. CTLA-4, which binds CD80 and inhibits dendritic cell activation [28], resulted in an immediate remission in a child with MCD [29]. Genetic polymorphisms of CTLA-4 have also been associated with the development of MCD [30, 31].

Given that CD80 has also been hypothesized in the development of other glomerular diseases such as FSGS and diabetic nephropathy [32, 33], its specific role in MCD remains controversial, but these observations strongly suggest that podocyte CD80 activation could represent a response of the podocyte to an antigen, allergen, or hypoxic stimulus. We have also found elevated levels of urinary CD80 excretion in a patient presenting with nephrotic syndrome following bone marrow transplant, as well as in a few patients with posttransplant FSGS (unpublished). Urinary CD80 excretion also appears to be higher in MCD than in FSGS or other glomerular diseases [23, 24, 29, 34], and based upon these observations, we and others have hypothesized that proteinuria may involve a two-hit disorder, in which podocyte CD80 is induced by a virus or allergen but then its expression continues due to an impaired CTLA4 response [35–37]. The administration of CTLA4-Ig has previously been reported to be beneficial in patients with posttransplant FSGS who express CD80 in glomeruli [33]. However, these patients also received plasma exchange along with other immunosuppressive agents and the decrease in proteinuria cannot solely be attributed to CTLA-4-Ig administration [38]. Indeed, some FSGS patients experience overwhelming proteinuria despite normal levels of urinary CD80 suggesting that the mechanism of proteinuria in FSGS is not CD80-driven when compared to MCD.

Our group has also recently discovered that the xenograft nephropathy, the nephrotic syndrome associated following xenotransplantation, is also associated with the induction of CD80 expression in podocytes. In particular, we found that the nephrotic syndrome observed in baboon recipients of pig xenografts has (i) increased urinary CD80 excretion that precedes the development of proteinuria (the urinary CD80 appears to be of both baboon and porcine origin), (ii) CD80 was found to be expressed in glomeruli by immunostaining in biopsies of baboons with nephrotic syndrome, and (iii) multiple doses of CTLA4-Ig therapy added on top of an anti-CD40L-based regimen resulted in a marked reduction in proteinuria with significantly improved survival compared to baboons treated with the anti-CD40L-based regimen without the CTLA4-Ig therapy (Yamada et al., manuscript in preparation). By minimizing proteinuria with this modified protocol, we have recently achieved >6 months of stable renal function in baboon recipients of GalT-KO pig thymokidneys without additional gene modifications [8]. This 193-day life-supporting kidney xenograft recipient is thus far the longest known survival of a GalT-KO kidney without additional gene modifications in a baboon. Most notably, the recipient had unresponsive in vitro pig-specific cellular assays and did not develop elicited baboon antipig antibodies.

2.1.4. Recent Progress Using Multitransgene Donors. Although initial studies demonstrated that GalT-KO kidneys without vascularized thymic grafts survived for 34 days in baboons [3], recently published data from the Pittsburgh group has shown that the use of multi-Tg hCD46/hCD55/EPCR/hCD39 GalT-KO pig donors allows for survival of a life-supporting kidney for >6 months in baboons [6]. These results suggest the beneficial effects of multigene editing for prolonging xenograft survival. Despite these promising results, however, similar attempts by other with hCD39/hCD55/hCD59 and α1,2-fucosyltransferase GalT-KO pig kidneys in cynomolgus macaques clearly demonstrated the development of proteinuria [10] indicating that the exact responsible genes have yet to be identified.

Recently, incompatibilities between porcine CD47 and the baboon signal regulatory protein α (SIRP-α), an interspecies ligand-receptor responsible for the activation of macrophages and phagocytosis in xenogeneic combinations, have been identified [39–42] (Figure 1). Immune activation of the porcine podocyte leads to expression of CD80 which potentially downregulates SIRP-α and SMPDL-3b. The exact role of hCD47 on the development of proteinuria in vitro as well as in vivo using transgenic hCD47-GalT-KO pigs [43] is currently being investigated in our laboratories with preliminary results demonstrating that SIRP-α expression is markedly decreased following xenotransplantation of GalT-KO kidneys in baboon that develop proteinuria and that the addition and high expression of hCD47, as well as the addition of hDAF transgenes to GalT-KO pig donors, nearly eliminated proteinuria following thymokidney transplantation in baboons (Yamada et al., manuscript in preparation).

3. Differences in Kidney Size May Affect Outcomes in Xenotransplantation

3.1. Organ Growth Discrepancies. As the xenotransplant community continues to make steady progress in overcoming immunological barriers, new concerns such as limitations due to growth discrepancies from varying donor strains have risen. Previous work from our laboratory has

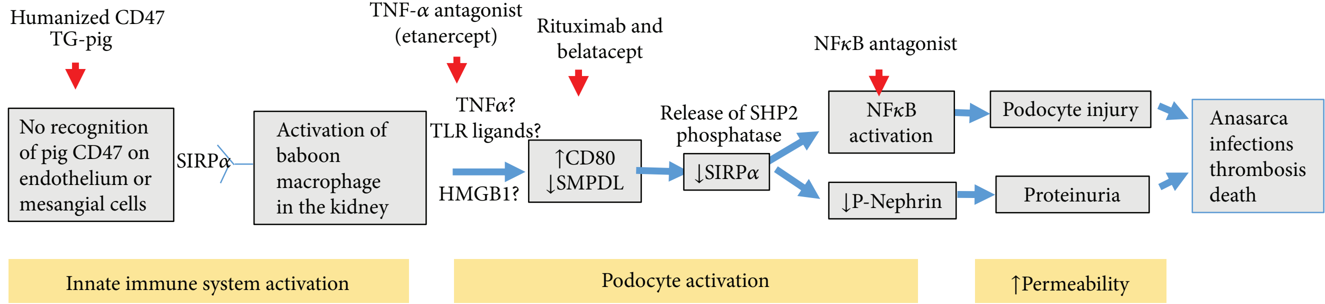

Figure 1: Schematic diagram of potential mechanisms of development of proteinuria. Proposed pathway and causative mechanisms for the development of proteinuria following pig-to-NHP kidney xenotransplantation.

clearly demonstrated discrepancies in the rate of kidney growth following transplantation utilizing varying donor strains [8]. The importance of matching donors/recipient pairs will become paramount when considering xenotransplantation in special populations, such as pediatric and adolescent recipients, where rapid growth in a limited cavity could cause graft dysfunction. Early attempts to study organ growth following xenotransplantation were led by Soin et al. [44]. The authors have studied 6 cases of pig-to-NHP kidney xenotransplantation where survival was up to 48 days and organs grew for 2 weeks following transplantation, after which point growth plateaued in 3 recipients but continued to grow in the remaining 3 recipients and ultimately led to xenograft failure [44]. While kidneys markedly increased in size due to rejection, the nonrejected xenografts also grow (as judged by weight) at a rate similar to porcine kidneys in their native environment [44]. This natural increase in porcine graft size could lead to a local compression within a limited compartment (a "compartment syndrome" such as observed with the "Page kidney"), especially in pediatric recipients, which could be another barrier to successful xenotransplantation.

To better understand the role of continued growth on organ function and to address the question if organ growth is regulated by intrinsic (organ) factors of extrinsic (host) factors, our laboratory recently investigated the importance of the role of donor strain on organ growth following pig-to-pig kidney and lung allotransplantation and pig-to-baboon kidney xenotransplantation using outbred (Yorkshire) and inbred (MGH miniature swine) donors [8]. Following xenotransplantation, renal volumes were measured at routine intervals at which time was determined that if the ratio of the donor pig kidney volume to recipient body weight was greater than 25 cm^3/kg, there appeared to be compromise of renal function with eventual graft loss and further alluding to the possibility that growth appeared to be regulated by intrinsic factors. The likely explanation for this deleterious effect is that increased growth causes compromise in renal blood flow to the enlarging xenograft, which in turn leads to cortical ischemia, as the circulating blood volume in the recipient cannot perfuse the graft with enough volume. In addition, the recipient baboons have limited abdominal space that can accommodate a xenograft. As the graft continues to grow, there is eventual development of a compartment syndrome type of effect that further causes decline in renal function as a result of extrinsic compression [8].

In order for xenotransplantation to be clinically applicable, organ growth and size matching will need to be considered, especially in pediatric and petite patients, where continued organ growth from larger donors could cause graft compromise. Although wild type pigs are generally used for production of GalT-KO or multitransgenic GalT-KO pigs because of its productivity, these pigs grow much faster and can reach sizes > 300 kg. In order to more closely examine the morphology of porcine kidneys, Lazo et al. compared anatomical characteristics between 96 Landrace/Yorkshire (used in our previous studies as controls) and 60 Dalland swine which grew to an average of 95 kg and at just 5 months had renal volumes that matched average adult human renal volumes [45]. Despite being anatomically suitable for xenotransplantation, continued growth mismatch of these kidney breeds in human recipients could lead to organ dysfunction if the xenograft continues to undergo unregulated growth as we have previously demonstrated. Miniature swine instead are generally considered the best match for potential human xenotransplantation because of their size, but also because of largely known genetic profile [1, 46–48]. Though early, further research in this field is warranted and currently ongoing to elicit specific factors responsible for continued growth which could be the target of gene therapy [49] in an effort to eliminate this phenomenon.

4. Conclusions

The development of xenograft nephropathy following XKTx appears to be closely associated with upregulation of CD80 and loss of SMPDL-3b in porcine podocytes. These changes appear to be due in part to preformed natural antibodies, not elicited antipig antibodies. Additionally, interspecies incompatibilities between CD47 and SIRP-α seem to induce glomerular endothelial damages that potentially penetrate the glomerular network and ultimately lead to disruption and damage to porcine podocytes. As work in overcoming immunologic hurdles advances, it appears that additional mechanisms, such as discrepancies in organ growth from donors or varying sizes/strains, may play an important role.

Recent work seems to demonstrate that uninhibited growth of transplanted organs may lead to comprise of graft function. As a result of recent data, further work in understanding mechanisms for organ growth, as well as specific size cut-offs, is currently being investigated. Ultimately, as success in controlling posttransplant proteinuria, as well as the selection of appropriate donor strains occurs, XKTx may become a reality, providing patients eagerly waiting on the transplant list will a new avenue of hope.

Abbreviations

FSGS: Focal segmental glomerulosclerosis
GalT-KO: Alpha-1,3-galactosyltransferase knockout
HAR: Hyperacute rejection
h: Human
hDAF: Human decay-accelerating factor
MCD: Minimal change disease
MHC: Major histocompatibility complex
NHP: Nonhuman primates
PBL: Peripheral blood lymphocytes
POD: Postoperative day
SMPDL-3b: Sphingomyelin phosphodiesterase acid-like 3b
Tg: Transgenic
VTL: Vascularized thymic lobe
XKTx: Xenogeneic kidney transplantation.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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Research Article

Beneficial Effects of Human Mesenchymal Stromal Cells on Porcine Hepatocyte Viability and Albumin Secretion

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Porcine hepatocytes transplanted during acute liver failure might support metabolic functions until the diseased liver recovers its function. Here, we isolated high numbers of viable pig hepatocytes and evaluated hepatocyte functionality after encapsulation. We further investigated whether coculture and coencapsulation of hepatocytes with human multipotent mesenchymal stromal cells (MSC) are beneficial on hepatocyte function. Livers from 10 kg pigs ($n = 9$) were harvested, and hepatocytes were isolated from liver suspensions for microencapsulation using alginate and poly(ethylene-glycol)- (PEG-) grafted alginate hydrogels, either alone or in combination with MSC. Viability, albumin secretion, and diazepam catabolism of hepatocytes were measured for one week. $9.2 \pm 3.6 \times 10^9$ hepatocytes with $95.2 \pm 3.1\%$ viability were obtained after isolation. At day 3, free hepatocytes displayed 99% viability, whereas microencapsulation in alginate and PEG-grafted alginate decreased viability to 62% and 48%, respectively. Albumin secretion and diazepam catabolism occurred in free and microencapsulated hepatocytes. Coencapsulation of hepatocytes with MSC significantly improved viability and albumin secretion at days 4 and 8 ($p < 0.05$). Coculture with MSC significantly increased and prolonged albumin secretion. In conclusion, we established a protocol for isolation and microencapsulation of high numbers of viable pig hepatocytes and demonstrated that the presence of MSC is beneficial for the viability and function of porcine hepatocytes.

1. Introduction

Hepatocyte cell transplantation is a potential solution to temporarily improve acute liver failure in patients that are waiting for a liver transplant [1]. Nevertheless, for patients with acute liver failure, the availability of suitable human liver cells remains a major problem. Transplantation of xenogeneic cells could be an adequate cell replacement treatment [2]. To circumvent the immune reaction toward the xenogeneic cells, encapsulation of cells in a semipermeable biocompatible polymer has been developed [3]. Micropores permit exchanges of nutrients, oxygen, and small-sized molecules. The promising first transplants with encapsulated hepatocytes in pig-to-rodent animal models showed that such transplanted encapsulated hepatocytes were still functional over several weeks [4–6]. In a pig-to-baboon model, 75% of baboons after induction of fulminant liver failure and subsequent transplantation with encapsulated hepatocytes recovered from liver injury [7], demonstrating further the therapeutic potential of such a treatment.

Previously, our laboratory established the first protocol for the isolation of primary pig and human hepatocytes and their encapsulation with alginate- (Alg-) poly-L-lysine (PLL) polymers [5, 6]. However, hepatocytes when encapsulated showed reduced functionality suggesting that polymers and microenvironmental conditions needed further optimization.

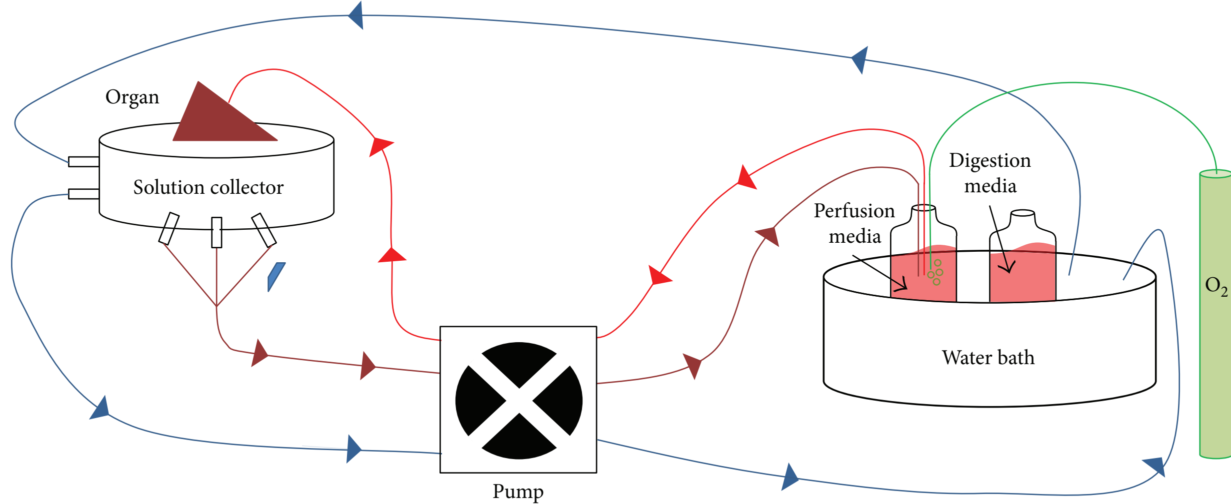

Figure 1: Liver perfusion and digestion system. The isolated liver is maintained at 37°C by water circulation (in blue) supplied by a water bath kept at 37°C. Perfusion and digestion media are maintained at 37°C and oxygenated through the oxygen cylinder (in green). Thereafter, infusion of perfusion media followed by digestion media (in red) into the liver is performed via the vena cava. Flow-through of both media (in brown) is recovered and discarded.

Indeed, neonatal pig hepatocytes showed increased viability and functionality when previously reaggregated [4] or when heparin or hepatocyte growth factor was added during the encapsulation procedure [8]. The addition of PLL and collagen during encapsulation in alginate microcapsules prolonged viability and functionality, as demonstrated for cells of the human hepatocellular carcinoma cell line HepG2/C3A [9]. Further, coculture or coencapsulation with other cell types such as endothelial progenitor cells or fibroblasts showed enhanced viability and function of rat hepatocytes [10, 11].

Multipotent mesenchymal stromal cells, also called mesenchymal stem cells (MSC), support the growth of rodent hepatocytes by favoring cellular adhesion *in vitro* [12, 13] and *in vivo* [14]. Several studies in rodents have shown that cotransplantation of free primary hepatocytes with rat MSC prolonged graft function with a decrease in alanine transaminase (ALT), aspartate transaminase (AST), and bilirubin [15–17]. Further, coculture of rodent hepatocytes with MSC improved albumin secretion and metabolic capacities of hepatocytes [15, 16, 18].

The aim of this study was to optimize hepatocyte isolation to reach high yields of viable porcine hepatocytes and to assess viability, albumin secretory capacities, and metabolic functions after *in vitro* culture and microencapsulation. Furthermore, we aimed to assess the effect of human MSC on free and coencapsulated porcine hepatocytes, with particular attention to the effect on viability, albumin secretion, and cell morphology of pig hepatocytes.

2. Materials and Methods

2.1. Animals and Liver Harvesting. Ten-kilogram pigs were purchased from a local pig farm (M. Stirnimann, Apples, Switzerland) and were shortly housed in the animal facility of the University of Geneva, in compliance with all cantonal dispositions. Animal research was performed following protocols approved by the Geneva cantonal veterinary authorities (license GE/79/15). Pigs were fasted overnight and premedicated with azaperone (1 mg/kg) and atropine (0.05 mg/kg) + dormicum (0.5 mg/kg) by intramuscular injection, prior to anesthesia with isoflurane, fentanyl (0.1 mg), and atracurium (1 mg/kg). After heparin injection (5000 U/L) and disinfection, an abdominal incision was performed, followed by gallbladder removal and perfusion with cold preservation medium IGL-1 (3.5 L) (Institut Georges Lopez, Lissieu, France) through the portal vein and the hepatic artery. A total hepatectomy was performed, and the liver was stored on ice for a maximum of 30 min prior to hepatocyte isolation.

2.2. Cell Isolation and Culture. Isolation of porcine hepatocytes was performed as previously described [5]. Briefly, the liver was perfused through the vena cava with liver perfusion medium for 15 minutes at 37°C (Life Technologies, Carlsbad, CA, USA); thereafter, digestion media containing collagenase NB 4 Standard Grade (3 g/L; Serva, Heidelberg, Germany) were infused for 25 minutes through the vena cava (Figure 1). Mechanical destruction and filtration of the liver through a 100 μm stainless steel mesh were performed to obtain a hepatocyte suspension, as previously described [5]. The hepatocyte cell suspension was washed twice using hepatocyte wash medium (Life Technologies) and centrifuged at 68g for 10 minutes; cells were counted and then cultured in DMEM/F12 (Life Technologies), supplemented with dexamethasone (0.4 μg/mL, Sigma-Aldrich, Buchs, Switzerland), insulin (0.02 E/U/mL, Novo Nordisk, Plainsboro, NJ, USA), apo-transferrin (5 μg/mL, Sigma), penicillin (100 IU/mL) and streptomycin (100 mg/mL) (P-S; Gibco-Thermo Fisher, Waltham, MA, USA), and 10% of autologous serum,

obtained after clotting and high-speed centrifugation of porcine blood.

MSC were isolated from the femoral head of the patients undergoing total hip replacement. MSC were characterized by surface receptor expression using FACS analysis and by their capacity to differentiate into osteoblasts, chondrocytes, and adipocytes as shown previously [19, 20]. All patients gave informed consent, and the experimental procedure was approved by the local ethical committee of the University Hospitals of Geneva (NAC 01-015). Briefly, after isolation, through mechanical destruction and Ficoll density gradient centrifugation, MSC were cultured in Iscove's modified Dulbecco's medium (Gibco-Thermo Fisher) with 10% fetal calf serum (Gibco-Thermo Fisher), P-S, and 10 ng/mL platelet-derived growth factor BB (PDGF-BB; PeproTech EC Ltd., London, UK). MSC were used from passages 2 to 5.

2.3. Polymer Synthesis. Sodium-Alg Kelton High Viscosity (lot number 61650A; $\eta = 813\ \mathrm{mL{\cdot}g^{-1}}$ in 0.1 M NaCl, $T = 25^{\circ}\mathrm{C}$, and G/M = 0.6) was obtained from Kelco (San Diego, CA, USA). Commercial reagents (Fluka, Sigma, Switzerland; TCI Europe, Zwijndrecht, Belgium) were used without further purification. Unless mentioned otherwise, all reactions were performed under argon atmosphere (1 atm). Anhydrous solvents were obtained by filtration (PureSolv MD 5, Innovative Technology, Oldham, UK). Glassware was dried for 12 h in an oven ($T > 100^{\circ}\mathrm{C}$) or under vacuum with a heat gun ($T > 200^{\circ}\mathrm{C}$). Nuclear magnetic resonance (NMR) spectra were recorded on Bruker Avance III-400, Bruker Avance-400, or Bruker DRX-400 spectrometers at room temperature (rt) (400 MHz) (Bruker, Billerica, MA, USA). ^{1}H frequency is at 400.13 MHz. Chemical shifts are expressed in parts per million (ppm) and coupling constants (J) in hertz (Hz). The solvent used for NMR spectroscopy was deuterated water.

Polymer Alg-poly(ethylene-glycol)- (PEG-) SH (Alg-PEG-SH) was prepared according to a procedure that we recently established [21]. A solution of thiobarbituric acid-(TBA-) Alg [22] (200 mg, 0.478 mmol) in dimethyl sulfoxide (DMSO) (40 mL) was stirred for 12 hours at 22°C. To this solution, 1,1′-carbonyldiimidazole (CDI) (77.4 mg, 0.478 mmol) was added, and the mixture was stirred at 22°C for 0.5 h. Acetone (80 mL) was added, and the resulting precipitate was filtered and washed with acetone (20 mL, 3 times). The solid was dried for 15 minutes under vacuum at 40°C and dissolved in distilled water (20 mL). A solution of α-amino-ω-azido PEG (H_2N-PEG-SH) (98.8 mg, 95.8 μmol) in distilled water (1 mL) was added, and the solution was stirred for 2 hours at 22°C. NaOH (0.05 M aqueous solution) was added until reaching pH 11.0, and the solution was transferred to a dialysis membrane and dialyzed against water. After one water change, tris(2-carboxyethyl)phosphine (TCEP) (0.1 M, 1 mL) was added in the dialysis tube, and the dialysis was continued against water for 3 days. An aqueous solution of $NaHCO_3$ was added until reaching pH 7. The solution was filtered (70 μm and 0.22 μm) and freeze-dried to afford Alg-PEG-SH as a white solid (106.7 mg) (Supplementary Figure 1). The percentage of grafting, determined by H-NMR, was 30.3%. The viscosity of the polymer (measured in distilled water at 22°C) was 204.6 mPa·s.

2.4. Hepatocyte Microencapsulation. Hepatocytes and MSC were coencapsulated in a ratio 1 : 1, keeping in mind that hepatocytes are in contact with nonparenchymal cells in liver plates [23]. Hepatocytes (3×10^6/mL of polymer) with or without MSC (3×10^6/mL of polymer) were gently mixed with calcium-alginate (Ca-Alg), under sterile conditions. Alg-PEG-SH (3% diluted in 3-(N-morpholino)propanesulfonic acid (MOPS)) was also used to encapsulate hepatocytes. All polymers were prepared by collaborators of the École Polytechnique Fédérale de Lausanne. Microbeads were produced using the Buchi Encapsulator B-395 Pro (Büchi Labortechnik AG, Flawil, Switzerland). Ionic and covalent cross-linking occurred immediately after bead immersion into the gelation bath, composed of 10 mM MOPS with pH = 7.4 and 100 mM $CaCl_2$. After formation, microspheres were straightaway collected by filtration to remove the gelation bath, washed with 0.9% NaCl, and immediately cultured in complete DMEM/F12 medium. The diameter of resulting beads was 500–600 μm for Ca-Alg and 400–500 μm for Alg-PEG-SH [21].

2.5. Viability of Primary and Encapsulated Cells. Viability and cell death of free and microencapsulated cells (0.2×10^6 cells/1 mL/24-well plate) were analyzed using fluorescein diacetate (FDA) and propidium iodide (PI) for the staining of viable and dead cells, respectively, as previously described [24]. Images were acquired using a fluorescent microscope and LAS V4.5 software (Leica Microsystem, Heerbrugg, Switzerland). Quantification of cell viability was performed 3 days after culture using ImageJ (https://imagej.nih.gov/ij) and expressed as a percentage where the sum of the value measured, for FDA-positive and PI-positive cells in the field of view, was set as 100%.

2.6. Measurement of Albumin Secretion. Hepatocytes (0.2×10^6 cells) alone or with MSC (0.2×10^6 cells, ratio 1 : 1) and microencapsulated or not were seeded with 1 mL complete DMEM/F12 medium in a 24-well Corning Primaria Cell Culture Multiwell Plate (Fisher Scientific, Hampton, NH, USA). Prior to albumin secretion assays, cells were washed and serum-starved overnight to remove pig albumin. Every 24 hours, medium was collected from day 2 to day 8; samples were frozen until albumin measurements. Albumin was measured following the manufacturer's instructions using an albumin pig ELISA kit (Abcam, Cambridge, UK).

2.7. Measurements of Drug Metabolism. 1 μg/mL of diazepam was added to free and encapsulated hepatocytes (0.2×10^6 cells/1 mL/24-well plate) for 6 hours at days 1, 3, and 7. Supernatants were collected and frozen until metabolite measurements. Prior to the quantitative analysis, protein precipitation was performed on the samples using a solution of methanol : ethanol at a ratio 1 : 1. Then, samples were centrifuged at 14,000g for 15 minutes. Samples were lyophilized with a SpeedVac system and reconstituted in 10% methanol. Quantitative analysis was performed by LC-MS/MS with a selected reaction monitoring (SRM) mode. The UltiMate

3000 LC system from Dionex coupled to a triple quadripole 5500 QTRAP system from AB Sciex was used. The LC separation was conducted on a Kinetex C18 column (50×2.1 mm (i.d.)) (Phenomenex). The mobile phases were made of A (H_2O with 0.1% formic acid) and B (ACN + 0.1% formic acid). The flow rate was 0.6 mL/min. The SRM transitions used for the quantification of the diazepam and its respective metabolites are referred in Supplementary Table 1.

2.8. Immunofluorescence Staining on Cultured Cells. Hepatocytes (0.2×10^6 cells) alone or with MSC (0.2×10^6 cells, ratio 1 : 1) were seeded on 12 mm coverslips in a 24-well plate in 1 mL of complete F12 medium. After 3 days, cells were washed with phosphate-buffered saline (PBS) and fixed with a 10% formalin solution (Sigma-Aldrich, Buchs, Switzerland) for 12 min. Cells were then permeabilized with Triton X-100 0.1% diluted in PBS for 15 min, and epitopes were blocked using 0.5% bovine serum albumin (BSA) for 30 min. Hepatocytes were stained with an anti-pig albumin antibody (Abcam) diluted to 1/200 and a secondary Alexa Fluor 555 goat anti-rabbit antibody (Life Technologies) diluted to 1/500. MSC were stained with a mouse anti-human vimentin antibody diluted to 1 : 50 (Dako, Glostrup, Denmark) and a secondary Alexa Fluor 488 goat anti-mouse antibody (Life Technologies). For 5-ethynyl-2′-deoxyuridine (Edu) staining, the Click-iT EdU Alexa Fluor 488 Imaging Kit (Thermo Fisher) was used following the manufacturer's instructions. Coverslips were mounted using VECTASHIELD mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Cambridgeshire, UK). Images were acquired using a fluorescence microscope and LAS V4.5 software (Leica Microsystems).

2.9. Measurements of Cytokines in Cell Culture Supernatants. MSC alone or with hepatocytes (0.2×10^6 cells/1 mL/24-well plate) from 2 different donors were cultured in complete hepatocyte medium for 2 days. Cells were washed and cultured for 24 h in medium without FCS. Cell culture supernatants were collected and frozen until cytokine measurements. Cytokines were screened using Proteome Profiler, Human Cytokine Array (R&D Systems, Bio-techne, Minneapolis, MN, USA), following the manufacturer's procedures. Quantification of cytokine measurements was performed using ImageJ (https://imagej.nih.gov/ij) and expressed as mean pixel density.

2.10. Statistical Analysis. Results are expressed as mean ± standard error of the mean (SEM). Numbers of experiments are indicated in the legend of each figure. GraphPad Prism software was used. Values of hepatocytes alone or cocultured with MSC were compared using the ratio paired *t*-test, and differences were considered significant when $p < 0.05$ ($*$), $p < 0.01$ ($**$), and $p < 0.001$ ($***$).

3. Results

3.1. Hepatocyte Isolation and Cell Yield and Viability. After surgical recovery of the livers from 10-kilogram pigs ($n = 12$), $9.2 \pm 3.6 \times 10^9$ total hepatocytes were isolated with a yield of $27.9 \pm 9.9 \times 10^6$ cells/g (Table 1). Immediately after hepatocyte isolation and purification, viability was of 95.2 ± 3.1%, showing that the harvesting protocol allowed the isolation of high yields of viable porcine hepatocytes from 10-kilogram pigs. For the ultimate purpose to use frozen-stored porcine hepatocytes for transplantation, several batches of hepatocytes (10×10^6 hepatocytes in 1 mL of cryopreservation media) were frozen in 10% DMSO and ready-to-use CryoStor CS10 (Sigma-Aldrich) cryopreservation media. CryoStor-frozen cells were thawed in complete medium. After cell centrifugation at 68*g*, pellets of cells were resuspended in complete media. Cells were stained with trypan blue, and viability was evaluated under bright-field microscopy. 0.9×10^6 hepatocytes were obtained, corresponding to 90% of total cells. After seeding and culture, hepatocytes maintained viability at 3 days (Figure 2(a)); however, no viable hepatocytes were recovered when frozen with DMSO-containing medium. Furthermore, thawed hepatocytes after freezing in CryoStor maintained albumin secretion at days 4, 8, and 11 (Figure 2(b)).

Table 1: Hepatocyte isolation.

Total number of hepatocytes	$9.2 \pm 3.6 \times 10^9$
Cells/g	$27.9 \pm 9.9 \times 10^6$
Viability	95.2 ± 3.1

3.2. Hepatocyte Viability Is Maintained after Ca-Alg and Alg-PEG-SH Microencapsulation. During the *in vitro* culture of free and encapsulated hepatocytes, we assessed hepatocyte viability and mortality, using FDA-PI staining each day. *In vitro*, free hepatocytes maintained viability up to 10 days in adherent culture conditions; Alg- and Alg-PEG-SH-microencapsulated hepatocytes remained viable up to 7 days. After 3 days of culture, free hepatocytes were 99% viable with minimal cell death (1%; Figures 3(a) and 3(b)). After microencapsulation in beads of 400–600 μm diameters, Alg-microencapsulated hepatocytes maintained 62% viability and Alg-PEG-SH-encapsulated hepatocytes maintained 56% viability (Figures 3(a) and 3(b)). These results demonstrate that microencapsulation of porcine hepatocytes with both types of polymers allows survival of up to 50% of the microencapsulated pig hepatocytes.

3.3. Albumin Secretion Is Maintained in Hepatocytes after Microencapsulation. To assess hepatocyte functionality after microencapsulation, albumin secretion was measured starting from day 2 to day 8 in free, Alg-microencapsulated, and Alg-PEG-SH-microencapsulated hepatocytes. Measurements at 24 hours showed that free hepatocytes secreted 10–12 μg/mL/24 hours albumin until day 6, and at days 7 and 8, albumin secretion was halved (Figure 4(a)). The total amount of albumin secreted during 8 days was around 46 μg/mL in free cultured hepatocytes (Figure 4(a), grey bar). The albumin secretion from free hepatocytes isolated from 9 livers was at similar levels. Alg-microencapsulated hepatocytes secreted lower amounts of albumin (2–1.5 μg/mL/24 h), with a maximum amount measured at day 4 to day 6. Despite the

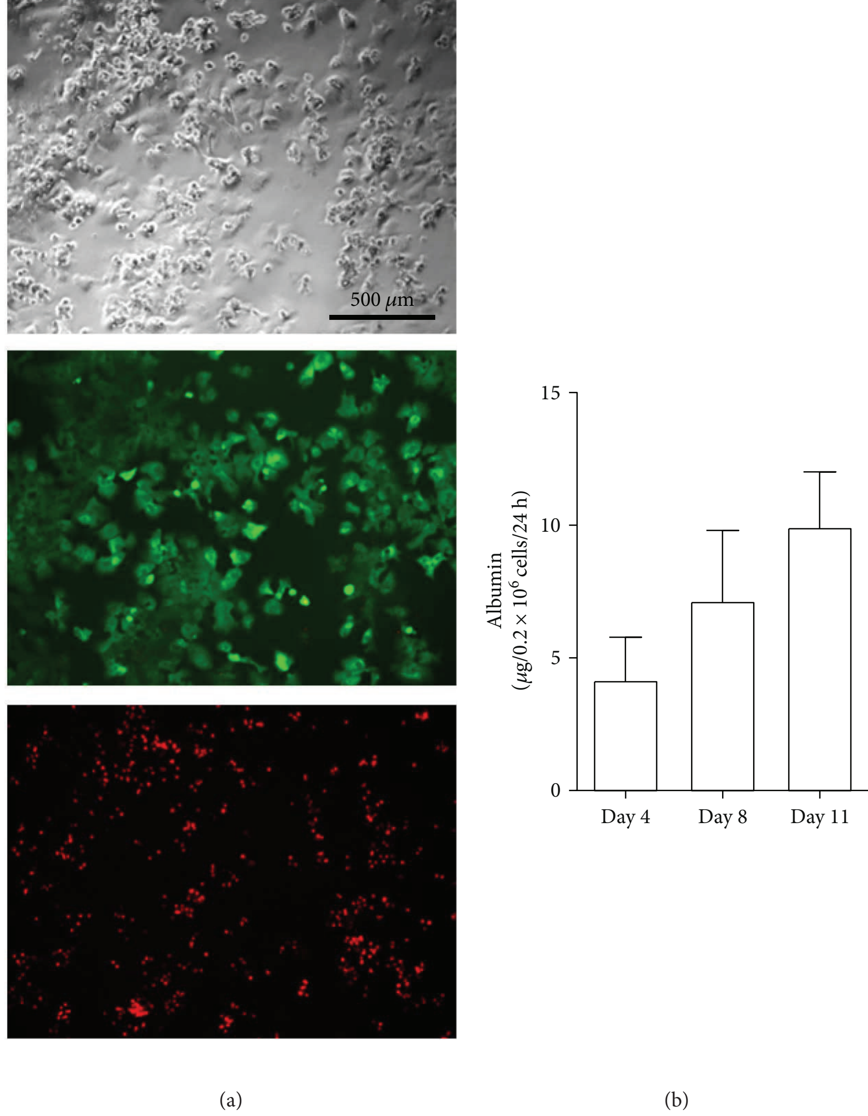

Figure 2: Hepatocyte viability and function after freezing and thawing. (a) The upper panel presents the bright-field image of thawed hepatocytes in culture after 3 days, the middle panel presents viable cells (FDA staining), and the lower panel presents nonviable cells (PI staining). (b) Albumin was measured by ELISA in the supernatant of cultured hepatocytes at days 4, 8, and 11. The experiment is performed in duplicates.

decrease in the amount of albumin measured, secretion still persisted until day 8 (Figure 4(b)). Alg-PEG-SH-microencapsulated hepatocytes secreted albumin until day 8 in similar amounts when compared with free cultured hepatocytes (Figure 4(c)), with the highest secretion at day 6. These results show that microencapsulated hepatocytes secrete albumin, which is maintained up to 8 days in free, Alg-microencapsulated, and Alg-PEG-SH-microencapsulated hepatocytes.

3.4. Microencapsulated Hepatocytes Still Display the Capacity to Metabolize Diazepam. To evaluate the metabolic capacities of free and microencapsulated hepatocytes, diazepam was added at days 1, 3, and 7 to hepatocytes for 6 hours and the metabolites nordiazepam and temazepam were measured. As shown in Figure 5, diazepam decreased similarly in both free and Alg-microencapsulated hepatocytes (white bars). At day 1, metabolic capacities were maximal; nordiazepam and temazepam were 49 ng/mL and 93.4 ng/mL, respectively, in free hepatocytes and 3.5 ng/mL and 5.6 ng/mL, respectively, in microencapsulated hepatocytes. The amounts of metabolites released decreased progressively between day 3 and 7, for both free and Alg-microencapsulated cells. This result suggests that, although lower amounts of diazepam metabolites were measured in the supernatant of encapsulated hepatocytes, hepatic metabolic function for diazepam still occurred in microencapsulated hepatocytes during the first week of *in vitro* culture.

3.5. Hepatocytes Show Increased Viability and Albumin Secretion When Coencapsulated with MSC. Trophic molecules secreted by MSC have been described to be beneficial

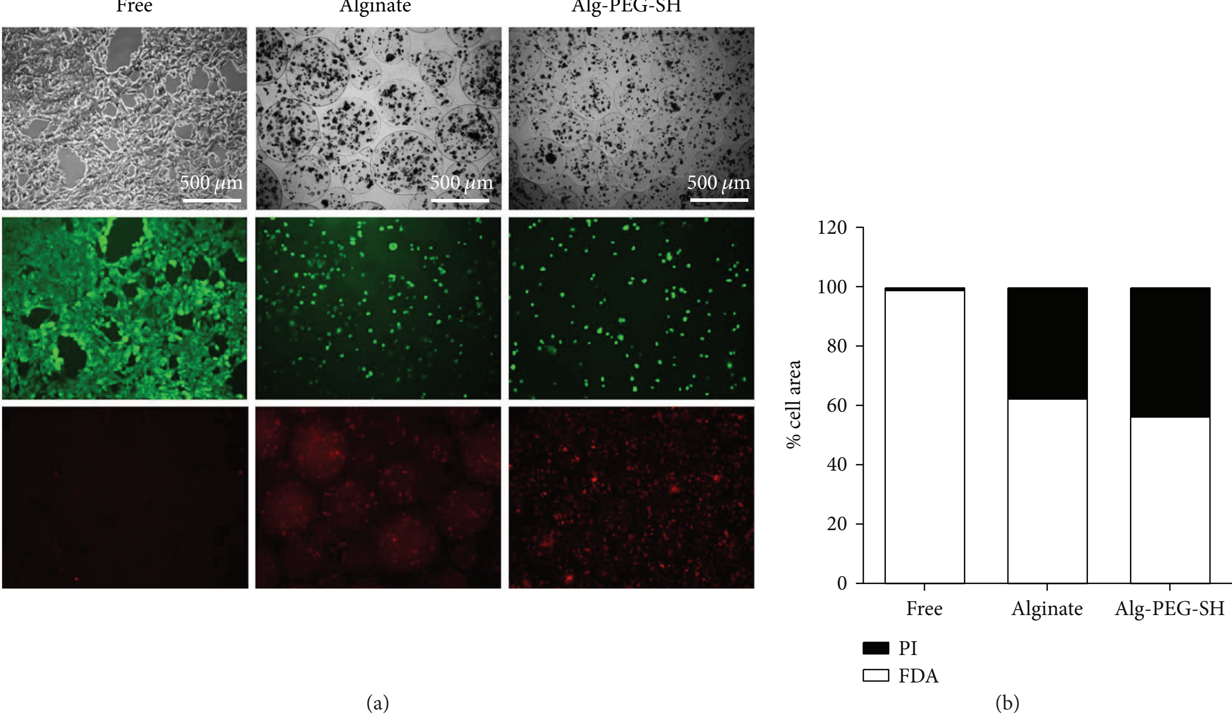

Figure 3: Viability of free and microencapsulated hepatocytes. (a) Representative images at day 3 for free, Alg-microencapsulated, and Alg-PEG-SH-microencapsulated hepatocytes. Upper panels present bright-field images, middle panels present viable cells (FDA staining), and lower panels present nonviable cells (PI staining). (b) Quantification of viable cells (FDA) and nonviable cells (PI) at day 3. Values are expressed as % of the total cell area ($n = 6$). Quantification has been performed using ImageJ.

for cell function in acute liver failure [25]. To assess whether MSC could provide a beneficial effect on microencapsulated hepatocytes, both were coencapsulated in an Alg microsphere. To assess viability, FDA-PI staining was performed at day 3. We observed that in capsules containing hepatocytes and MSC, total cell death was decreased considering the total number of PI- and FDA-stained cells. Hepatocyte survival reached 90% whereas hepatocytes alone reached 40% (Figures 6(a) and 6(b)). Further, to assess the functionality of hepatocytes coencapsulated with MSC, albumin secretion was measured each day starting at day 2 until day 8. In the presence of MSC (grey bars), albumin secretion was significantly higher at days 4, 5, and 8 ($p < 0.05$, Figure 7(a)) for hepatocytes coencapsulated with MSC than for microencapsulated hepatocytes alone (white bars). To assess the importance of cellular contact between hepatocytes and MSC, coculture was performed and albumin secretion was measured in the supernatants. At days 3 and 4, albumin secretion was significantly higher in hepatocytes with the presence of MSC (grey bars, $p < 0.001$) compared to hepatocytes alone (Figure 7(b), white bars). Noteworthy, MSC alone secreted only limited amounts of albumin (grey bars). Further, the presence of MSC allowed prolonging albumin secretion up to day 15 (Supplementary Figure 2). Diazepam metabolism of hepatocytes cocultured with MSC was similar to that of hepatocytes cultured alone (Supplementary Figure 3), suggesting that the cell-cell contact between hepatocytes and MSC does not affect metabolic function of hepatocytes at short time. All together, these results show that coencapsulation of hepatocytes with MSC improves and prolongs significantly albumin secretion from porcine hepatocytes.

3.6. Human MSC and Pig Hepatocyte Distribution in Coculture. Coculture of hepatocytes and MSC was used to analyze the cell distribution after 3 days of culture. MSC were stained for the cytoskeleton protein vimentin (in green) and hepatocytes for albumin (in red) (Figure 8(a)). Hepatocytes alone formed a typical adherent epithelial cell layer (Figure 8(a)). In coculture with MSC, hepatocytes appeared in small cell clusters (hepatocyte doublets or triplets), with the presence of MSC intermingled throughout the hepatocyte culture (Figures 8(b) and 8(c)).

3.7. Cytokine Screening in Supernatants of Human MSC Cultured Alone or with Porcine Hepatocytes. Cytokines secreted from MSC alone or cocultured with hepatocytes were screened using a human antibody array to evaluate an eventual cytokine response from MSC induced by hepatocytes. Several cytokines were detected in the 24 h supernatants (Figure 9(a)). CCL2, CXCL12, and macrophage migration inhibitory factor (MIF) were present in both cell culture supernatants. CXCL1 and IL-8 were only detectable in the supernatant of MSC from 1 of 2 donors. Interestingly, serpin E1 was present in the supernatants of MSC cultured alone, but its presence was significantly increased in MSC cocultured with hepatocytes (Figure 9(b)). These results show that MSC secrete only few inflammatory cytokines

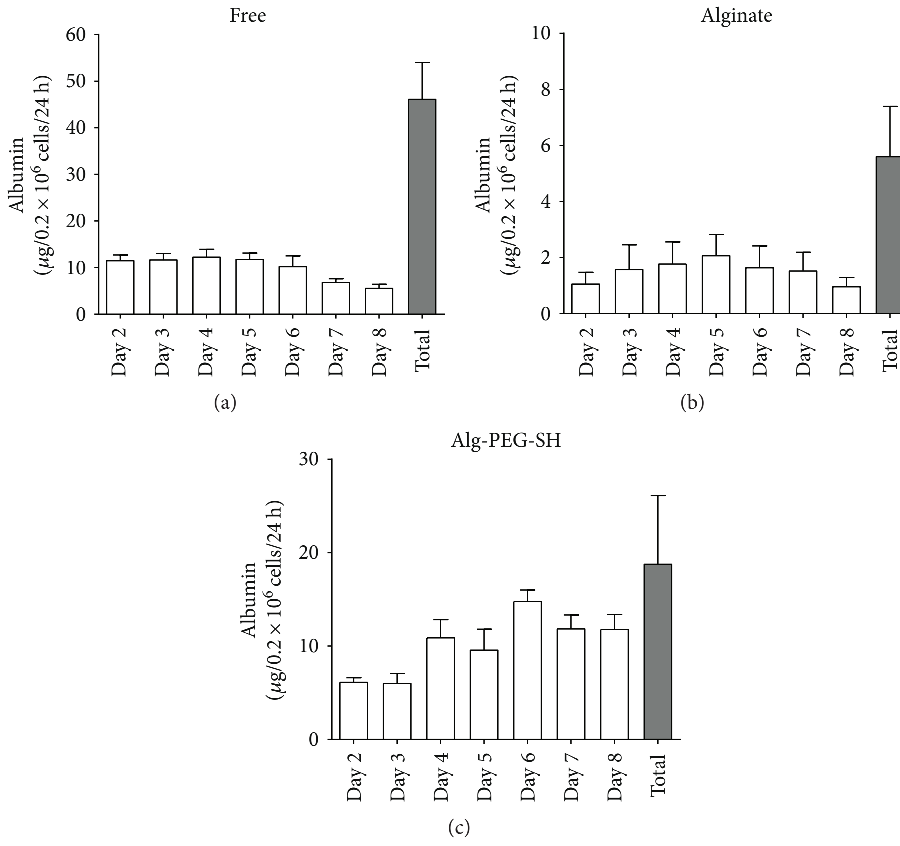

Figure 4: Albumin secretion from free and microencapsulated hepatocytes. Albumin was measured by ELISA in the supernatant of free ($n = 9$) (a), alginate-encapsulated ($n = 3$) (b), and Alg-PEG-SH-encapsulated ($n = 3$) (c) hepatocytes. White bars represent albumin secretion during 24 h from day 2 to day 8, and grey bars represent the total albumin secreted during 8 days.

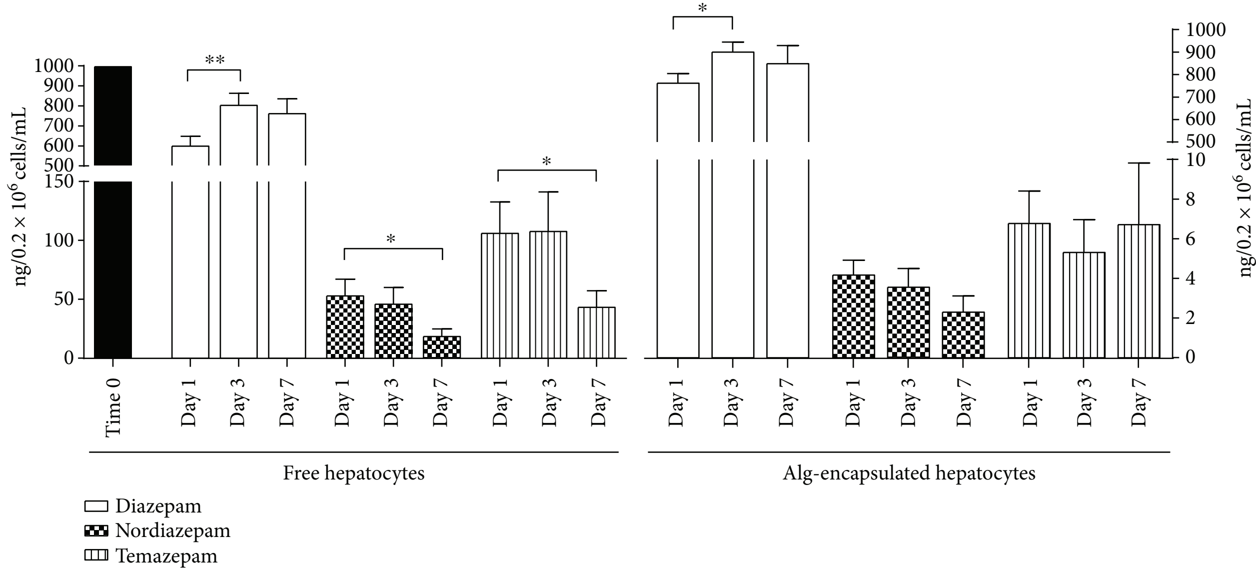

Figure 5: Diazepam metabolism in free and alginate-microencapsulated hepatocytes. Diazepam (white bars) was added at days 1, 3, and 7 on free and alginate-encapsulated hepatocytes, and supernatant was retrieved after 6 hours of culture. Diazepam and its metabolites nordiazepam and temazepam were measured by LC-MS/MS in 4 independent experiments. $^*p < 0.05$, $^{**}p < 0.01$.

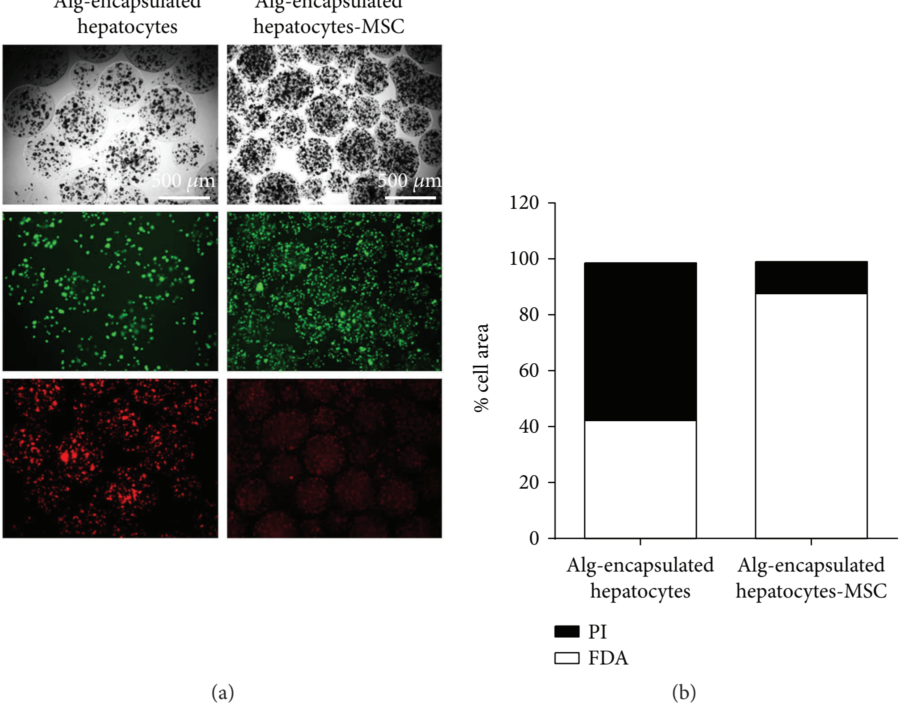

FIGURE 6: Viability of coencapsulated hepatocytes and MSC. (a) Representative images of Alg-encapsulated hepatocytes alone or with MSC after 4 days of culture. Upper panels present bright-field images, middle panels present viable cells (FDA staining), and lower panels present nonviable cells (PI staining). (b) Quantification at day 3. Values are expressed as % of the total cell area ($n = 2$). Quantification has been performed using ImageJ.

and this also occurs during coculture with porcine hepatocytes suggesting further that MSC do not increase the expression of proinflammatory cytokines when in contact with porcine hepatocytes at a short term.

4. Discussion

Acute liver failure has high mortality rate. Liver transplantation, which has to occur in the few days following liver destruction, remains the only treatment with lifelong immunosuppression thereafter [1]. Additionally, the availability of an adequate human liver is not guaranteed, demonstrating the need for new therapeutic options. Porcine hepatocyte cell transplantation might present an alternative solution to overcome acute liver failure damages by replacing the metabolic function of the liver until its recovery. Earlier studies in mice with acetaminophen- and hepatectomy-induced fulminant liver failure showed that transplantation of microencapsulated human or porcine hepatocytes increased survival rates of mice [5, 6] and the first protocols for porcine hepatocyte isolation and encapsulation with Alg-PLL capsules were applied [5, 6].

Here, we developed an optimized high-yield porcine hepatocyte isolation protocol from 10-kilogram pigs. Hepatocytes were encapsulated in recently developed biomaterials which showed an improved biocompatibility, compared to previous polymers. Currently, new biocompatible biomaterials which allow the production of long-term stable microspheres for hepatocyte encapsulation are under investigation. Long-term stability remains an important issue to prevent immunoreaction due to microcapsule disaggregation. Several types of polymers are under investigation. Durkut and collaborators showed that rat hepatocytes maintained comparable viability in free and encapsulated conditions in Alg-chitosan-Alg microcapsules [26]. However, this study did not focus on microbead stability. Furthermore, another study reports that hepatocytes isolated from rats and encapsulated in PEG did not survive after encapsulation [11]. Therefore, improvements are needed to maintain viability of primary hepatocytes which are highly sensitive to environmental conditions. Our data shows that using Alg and a hybrid Alg-PEG-SH biomaterial for encapsulation allows maintaining 56% viability of encapsulated hepatocytes, compared to hepatocytes alone. The diminished hepatocyte viability was reflected by decreased albumin secretion and metabolic activity of diazepam, but the function was partially maintained after microencapsulation when compared to that of free cultured hepatocytes. Actually, the initial amount of diazepam decreased in free and encapsulated conditions; however, the quantity of metabolites did not significantly increase in the supernatants of encapsulated hepatocytes. An issue to be considered is that molecule diffusion might be delayed; indeed, albumin and diazepam and its metabolites may remain longer inside the microsphere, making it difficult to compare the amounts of molecules released after 6 hours from free and encapsulated hepatocytes; however, cell viability and molecule dispersion were maintained.

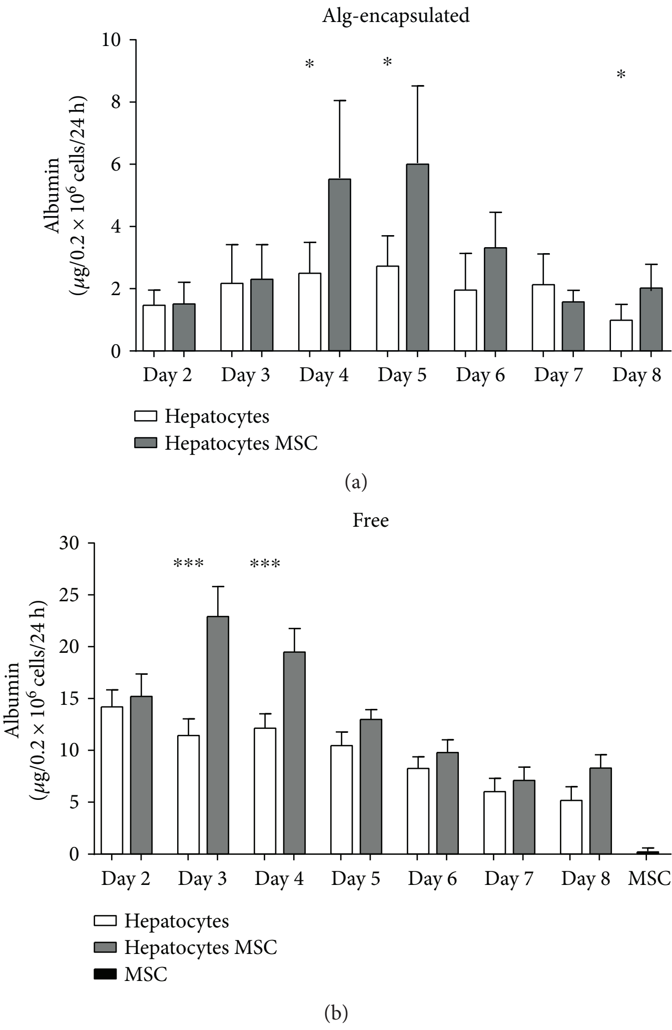

Figure 7: Albumin secretion of hepatocytes coencapsulated and cocultured with MSC. Albumin secretion was measured by ELISA in the supernatant of cell culture after 24 hours from day 2 to day 8. (a) Alg-microencapsulated hepatocytes alone (white bars) or with MSC (grey bars), measured in 2 independent experiments. (b) Free hepatocytes alone (white bars) or with MSC (grey bars), measured in 5 independent experiments. $^{*}p < 0.05$, $^{***}p < 0.001$.

A number of options to improve hepatocyte viability and function have been proposed. The supplementation with modulators like heparin or collagen during microencapsulation improves albumin and urea synthesis and increases hepatocyte viability [8, 9]. For rat hepatocytes, coencapsulation with other cells such as endothelial progenitor cells improves albumin and urea secretion [10]. MSC express low levels of human leukocyte antigen classes 1 and 2 and costimulatory molecules on their surface, conferring MSC low immunogenicity [27]. Furthermore, MSC have the capacity to suppress cell activation and proliferation of immune cells, in particular T cells, B cells, and dendritic cells [28]. The immune response of MSC to xenogeneic hepatocytes was analyzed using an antibody array for screening human cytokines. Under our culture conditions, with two different donor-derived MSC populations, we detected mainly 2 types of cytokines, CCL2 and MIF. CCL-2 and MIF are implicated in the chemotactic activity of immune cells and cell-mediated immunity, respectively [29, 30]. Furthermore, serpin E1 is increasingly detected in coculture conditions with hepatocytes. Serpin E1 is a serine protease inhibitor [31] secreted by MSC as demonstrated by Daltro et al., where serpin E1 secreted by MSC was involved in recovery of cardiac disturbances [32]. We did not observe major changes in the cytokine profile of MSC which further suggests that MSC, when exposed to xenogeneic hepatocytes, do not display a higher immunogenicity.

Moreover, MSC have antifibrotic properties [20] and have been successfully used to treat liver failure [33]. Therefore, we explored the effect of human MSC on porcine hepatocyte survival and function. The present results show that MSC significantly improve hepatocyte viability and albumin secretion in coculture and coencapsulated conditions. However, others have reported that for 3D cultures of human MSC and human hepatocytes, there was no effect on albumin secretion, but rather hepatocyte was compacted

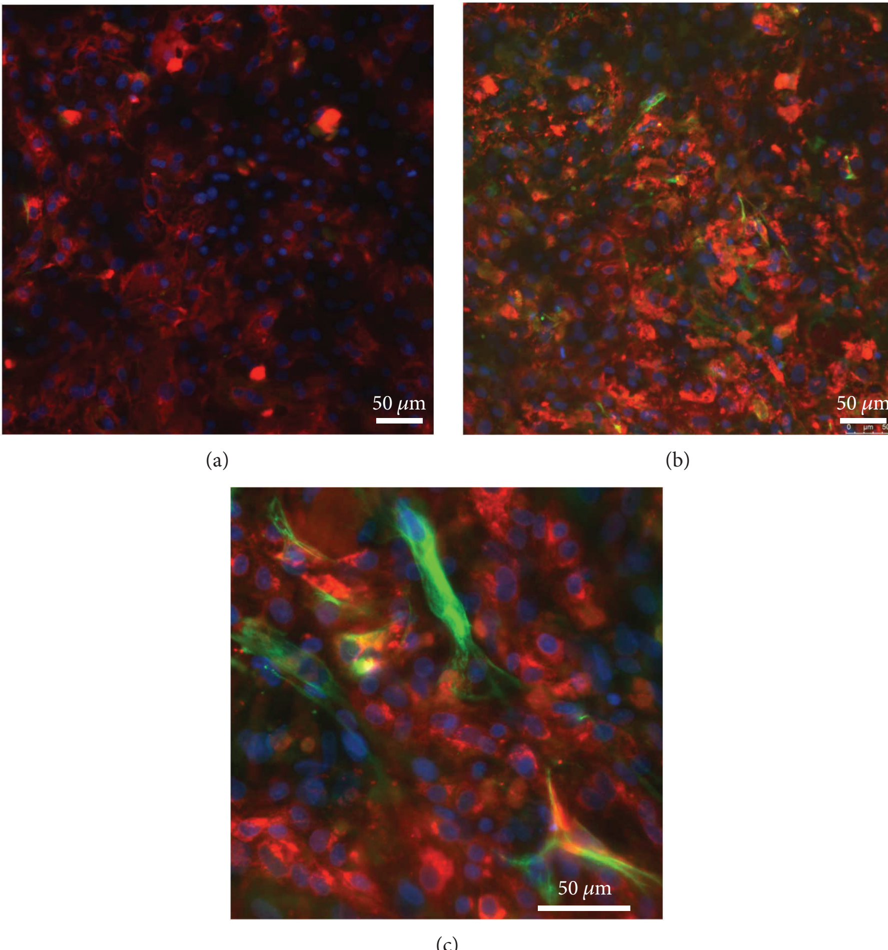

Figure 8: Distribution of porcine hepatocytes and human MSC in coculture. Cells were cultured for 2 days and then serum-starved to avoid nonspecific staining. Immunofluorescence was performed on cells after 3 days of culture. (a) Hepatocytes are stained for porcine albumin (in red). (b) Coculture of hepatocytes and MSC. MSC are stained for human vimentin (in green). (c) Coculture of hepatocytes and MSC in a higher magnification (×40). Images are representative of 2 independent pig hepatocyte isolations.

in morphology and there was a phenotypic stability [34]. Also, human MSC potentiated hepatotrophic and antiapoptotic genes in human primary hepatocytes [35], with the accumulation of hepatocytes in the G2/S phase of the cell cycle, meaning that they are prone to proliferation [18]. In line with these results, in the present study, hepatocytes in coculture with MSC tend to show extended survival compared to microencapsulated hepatocyte alone.

The mechanism how MSC exert their beneficial effects needs further investigations and might not solely be due to paracrine effects. Interestingly, the systemic injection of extracellular vesicles, derived from bone marrow MSC after *in vitro* culture, reduced hepatic injury and improved mice survival [25], suggesting that such vesicles contain molecules either acting directly on liver cells or modulating the immune system. Moreover, the evaluation of the secretome of human MSC evidenced a correlation between vascular endothelial growth factor and cell proliferation, development processes, and immune processes. Also, systemic injection of conditioned medium of MSC in liver-injured mice improved survival [36]. In our experimental conditions, we found increased albumin secretion when hepatocyte and MSC were cultured together; this might not be due exclusively to cellular interactions but might also include the effect of secreted molecules. In particular, in encapsulated condition, hepatocytes and MSC are distributed throughout the microcapsules (Figure 6(a)); both improved microenvironment and paracrine signaling through secreted molecules might contribute to the increased viability and functionality of hepatocytes.

5. Conclusion

In conclusion, we performed high-yield porcine hepatocyte isolations that allow obtaining high quantities of viable hepatocytes. Further, we used a newly developed polymeric biomaterial which allowed the maintenance of hepatocyte viability, albumin secretion, and drug metabolic functions up to 8 days. Furthermore, hepatocyte coencapsulation with MSC increased and prolonged hepatocyte viability, suggesting that cell-to-cell contact and paracrine effects are beneficial for hepatocyte function and survival. This optimized protocol for porcine hepatocyte isolation and microencapsulation can

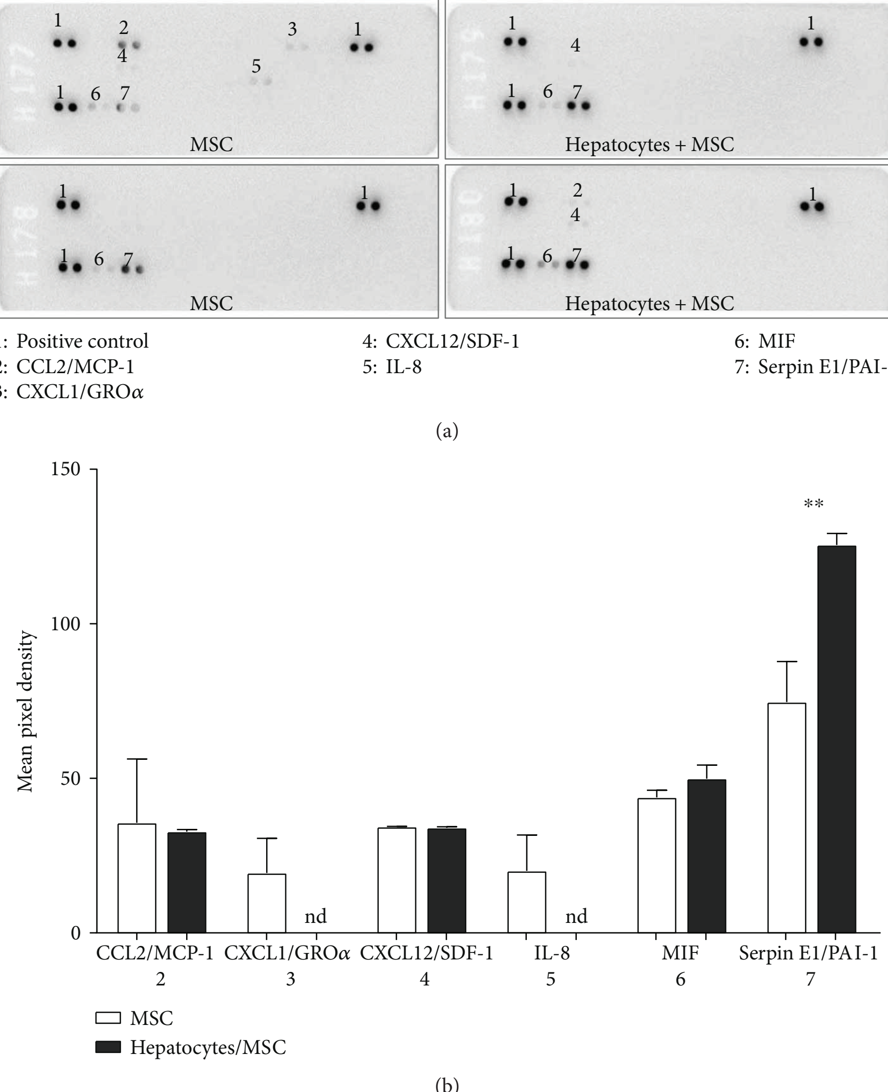

Figure 9: Profiling of human cytokines using cell culture supernatants of MSC alone and MSC cocultured with hepatocytes. (a) Human cytokines were analyzed using antibody membranes in MSC alone and MSC cocultured with hepatocytes (0.2×10^6 cells/mL/24-well plate) obtained from 2 different donors. (b) Spots were quantified using ImageJ and expressed as mean pixel density. Cytokines secreted from MSC cultured alone (white bars) and cytokines secreted from MSC cocultured with hepatocytes (black bars). $^{**}p < 0.01$.

now be used for further experimental cell transplantation research in liver diseases to evaluate further the potential of encapsulated hepatocytes for the treatment of acute liver failure in humans.

Abbreviations

Alg:	Alginate
Alg-PEG-SH:	Alginate-poly(ethylene-glycol)-SH
ALT:	Alanine transaminase
AST:	Aspartate transaminase
Ca-Alg:	Calcium-alginate
DMSO:	Dimethyl sulfoxide
Edu:	5-Ethynyl-2′-deoxyuridine
FDA:	Fluorescein diacetate
MIF:	Macrophage migration inhibitory factor
MSC:	Multipotent mesenchymal stromal cells
NMR:	Nuclear magnetic resonance
PBS:	Phosphate-buffered saline
PEG:	Poly(ethylene-glycol)
PI:	Propidium iodide
PLL:	Poly-L-lysine
SEM:	Standard error of the mean
SRM:	Selected reaction monitoring.

Disclosure

An earlier version of this work was presented as an abstract at the 14th Congress of the International-Xenotransplantation-Association (IXA), Baltimore, MA, September 20–23, 2017, and at the American Transplant Congress, 2017.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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Supplementary Materials

Supplementary 1. Supplementary Figure 1: polymer synthesis.

Supplementary 2. Supplementary Table 1: mean (±SD) yield and viability of hepatocytes isolated from 12 pigs.

Supplementary 3. Supplementary Figure 2: albumin secretion of hepatocytes cocultured with MSC until day 15. Albumin secretion was measured by ELISA in the supernatant of cell culture after 24 hours from day 2 to day 15. Free hepatocytes alone (white bars) or with MSC (grey bars), measured in 2 independent experiments.

Supplementary 4. Supplementary Figure 3: diazepam metabolism in hepatocytes cocultured with MSC. Diazepam was added at days 1, 3, and 7 and supernatant was retrieved after 6 hours of culture. Diazepam (white bars) and their metabolites nordiazepam, oxazepam, and temazepam were measured by LC-MS/MS in free hepatocytes and hepatocytes cocultured with MSC 4 independent experiments.

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Review Article

The Role of NK Cells in Pig-to-Human Xenotransplantation

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Recruitment of human NK cells to porcine tissues has been demonstrated in pig organs perfused ex vivo with human blood in the early 1990s. Subsequently, the molecular mechanisms leading to adhesion and cytotoxicity in human NK cell-porcine endothelial cell (pEC) interactions have been elucidated *in vitro* to identify targets for therapeutic interventions. Specific molecular strategies to overcome human anti-pig NK cell responses include (1) blocking of the molecular events leading to recruitment (chemotaxis, adhesion, and transmigration), (2) expression of human MHC class I molecules on pECs that inhibit NK cells, and (3) elimination or blocking of pig ligands for activating human NK receptors. The potential of cell-based strategies including tolerogenic dendritic cells (DC) and regulatory T cells (Treg) and the latest progress using transgenic pigs genetically modified to reduce xenogeneic NK cell responses are discussed. Finally, we present the status of phenotypic and functional characterization of nonhuman primate (NHP) NK cells, essential for studying their role in xenograft rejection using preclinical pig-to-NHP models, and summarize key advances and important perspectives for future research.

1. Introduction

The field of xenotransplantation explores the feasibility of replacing nonfunctional organs of one species by organs of another species and to overcome the current worldwide organ shortage in transplantation medicine [1]. Within the range of conceivable animals, pigs are the most suitable for xenotransplantation purposes for several reasons [2, 3]. However, before xenotransplantation becomes a clinical reality, many aspects of interspecies immunological and biological incompatibilities need to be taken into consideration [4, 5]. Recent reviews recapitulate the current advances in the field including a summary of the main mechanisms involved in xenorejection and how to control them and the longest survival times in pig-to-nonhuman primate (NHP) xenotransplantation models using transgenic pigs as donors, as well as the possibility of growing humanized organs in pigs using blastocyst complementation [6, 7].

A role for NK cells in the rejection of cross-species and allogeneic hematopoietic stem cell transplantation (hybrid resistance) was already reported in the 1980s [8, 9]. In contrast, the initiation and regulation of adaptive immune responses after solid organ transplantation by NK cells, promoting either rejection or tolerance, has been recognized only more recently [10–12]. As to xenotransplantation, the demonstration by Inverardi et al. of early xenogeneic cell-mediated events taking place at the interface between the endothelium of a discordant vascularized organ and the recipient's blood cells using *in vitro* experiments and ex vivo perfusion models has generated a particular interest in the role of NK cells [13, 14]. Following this inspiring and pioneering work performed during the early 1990s, several laboratories have studied the interactions of human NK cells and porcine endothelial cells (pECs) that result in endothelial cell activation and damage *in vitro*. In addition, an array of possible strategies to reduce the observed endothelial damage

caused by NK cells during rejection of vascularized xenografts has been put forward culminating so far in the generation of HLA-E transgenic pigs [15], which have been used in different xenotransplantation models [16–20].

During the past 20 years of research on NK cell biology, the view of these cells has evolved from simple killers to a heterogeneous, complexly regulated cell population able to control viral infections, to perform tumor surveillance, and to modulate adaptive immune responses [21–23]. Phenotypically human NK cells are characterized by the expression of the neuronal-cell adhesion molecule N-CAM (CD56) and the lack of CD3. Moreover, based on their level of CD56 expression, NK cells are divided into two major subpopulations: $CD56^{dim}$ NK cells, which are more cytotoxic and express high levels of the low affinity Fc-gamma receptor III A (FcγRIIIA, CD16); and $CD56^{bright}$ NK cells, which are characterized by the secretion of high levels of cytokines and low expression or absence of CD16 [24–26]. Overall, NK cell function is tightly regulated by the balance between activating and inhibitory signals mediated by a variety of NK cell receptors and their respective ligands on potential target cells. Upon recognition of "altered or abnormal cells" by one or a combination of the following mechanisms, these target cells will undergo lysis [27]:

(i) Recognition through CD16 (FcγRIIIA) of Abs bound to the surface of target cells leading to their elimination by a mechanism referred to as antibody-dependent cell-mediated cytotoxicity (ADCC).

(ii) Recognition of the lack of self-major histocompatibility complex (MHC) class I molecules on target cells by inhibitory NK cell receptors leading to direct NK cytotoxicity.

(iii) Presence of upregulated activating ligands on the surface of target cells (e.g., MICA/B for the activating NK receptor NKG2D) leading to direct NK cytotoxicity.

(iv) Interactions of FasL and TRAIL expressed on NK cells with Fas and TRAIL receptors expressed on target cells resulting in apoptosis of target cells.

All these potential mechanisms of activation, recognition, and elimination of target cells by NK cells, alone or in combination, induce the release of the content of their lytic granules (perforin, granzyme, and cytolysin). In addition, the production and secretion of proinflammatory cytokines, such as tumor necrosis factor (TNF) and interferon gamma (IFNγ), has a major impact on the shaping of adaptive immune responses. The ultimate goal of NK cell function is thus not only the specific destruction and removal of identified target cells but also the activation and/or regulation of other components of the cellular immune system [23, 25].

Several years have passed by since the last extensive reviews on the role of human NK cells in pig-to-human xenotransplantation [28–34]. We will therefore summarize our knowledge and update the recent advances accomplished, including the mechanisms behind recruitment of NK cells into xenogeneic organs and vascularized composite tissues perfused with human blood, the testing of therapeutic strategies designed to provide protection against recognition and destruction of xenografts by human NK cells, and how regulatory T cells and tolerogenic dendritic cells (DC) may modulate NK cell xenoreactivity.

2. Recruitment of Human NK Cells to Porcine Tissues

The endothelium forms the interface between the recipient blood circulation and the donor parenchyma and is therefore the first xenograft component encountered by the recipient immune system upon revascularization. NK cells infiltrate xenogeneic tissues as demonstrated in rat heart or pig kidney ex vivo perfusion models using human blood [13, 35], in guinea pig- and hamster-to-rat small animal xenotransplantation models [36, 37] and pig heart-to-baboon and pig kidney-to-cynomolgus large animal transplantation models [38–41]. However, more recent pig-to-NHP models using transgenic and/or KO pigs and different immunosuppressive protocols identified only small numbers of infiltrating NK cells [42]. In the pig-to-human combination, the perfusion of pig lungs and limbs with human blood lead to the sequestration of human NK cells [18, 20]. Less NK cell infiltration was noted in pig hearts during whole blood perfusions [19].

Human NK cells express several chemokine receptors which are active under physiological and under inflammatory conditions [43]. However, only little is currently known on the role of chemokines during recruitment of NK cells to xenografts. A mouse heart and islet allotransplantation model supported the notion that chemokines act via CXCR3 in the recruitment of lymphocyte subsets including NK cells [44]. On the other hand, Chen and collaborators showed the relevance of MCP-1 (also known as CCL2) in the recruitment of NK cells into concordant mouse-to-rat xenografts [45]. Nevertheless, rodent xenotransplantation models do not sufficiently mirror the species compatibilities of ligand-receptor interactions between human and pig. Contact of human whole blood with pECs led to the secretion of human IL-6, CCL3, CCL4, CCL5, CCL11, and CXCL8. Moreover, binding of human natural non-αGal xenoreactive Abs (XenoAbs) led to pEC activation and concomitant release of porcine chemokines and proinflammatory modulation of their surface receptors [46–49]. Chemokines such as CXCL8 secreted from activated pEC act on human polymorphonuclear neutrophils, preferentially through CXCR2 and PAF receptors, resulting in further secretion of chemokines and cytokines that can activate human NK cells [50].

Freshly isolated or IL2-activated, polyclonal NK cells were incubated with resting or activated, primary or immortalized pECs to further study NK cell responses against pECs. Various protocols were applied to analyze the different steps of recruitment *in vitro*, including under static and dynamic conditions simulating physiological shear stress [30, 51]. In our laboratory, primary and SV40-immortalized pECs derived from bone marrow (2A2) or aorta (PAEC,

PEDSV.15) were used. All cell lines constitutively express von Willebrand factor, LDL receptor, and swine leukocyte antigen (SLA) class I, while SLA class II is expressed upon pig IFNγ but not upon human IFNγ-, pig or human TNF-stimulation [52]. In addition, pECs express the following adhesion molecules: PECAM-1 (CD31), E-selectin (CD62E), P-selectin (CD62P), and vascular cell adhesion molecule-1 (VCAM-1, CD106) [52, 53].

Although the methodological differences make it somewhat difficult to directly compare the results obtained by different research groups, the most dominant receptor-ligand interactions for the recruitment of human NK cells to pECs have been elucidated (Table 1). Initial *in vitro* assays performed under static conditions demonstrated the ability of NK cells to adhere to both resting pECs as well as TNF-activated pECs [54–58]. These studies using peripheral blood mononuclear cells (PBMC) also demonstrated a role for interactions between human VLA-4 (CD49d/CD29) and porcine VCAM-1 (pVCAM-1), the importance of which was subsequently confirmed using purified human NK cells [59, 60]. An even more pronounced role of these molecules was later shown in assays under physiological shear stress [53] with specific blocking of either the human α4 integrin (CD49d) or pVCAM-1 resulting in 75% reduction of adhesion of freshly isolated or activated NK cells to pEC. A significant role was also demonstrated for the β2 integrin LFA-1, which is expressed on human NK cells, by using blocking antibodies against both subunits, CD11a and CD18, respectively [53, 60, 61]. In addition, human L-selectin (CD62L), which mediates rolling, was required for human NK cell adhesion to pECs under physiological shear stress [53]. Concurrent β2 integrin, VLA-4, VCAM-1, and L-selectin blockade completely inhibited lymphocyte attachment [62].

As to the transendothelial migration (TEM), an initial study by Hauzenberger et al. reported a strong reduction of human NK cell TEM across pEC monolayers when blocking pVCAM-1 [63]. Consequently, we could show a role for pVCAM-1 in the actual TEM by using a model that separates adhesion from TEM [64]. With the same model, it was also demonstrated that β2 integrin (CD18) blocking inhibits both adhesion and TEM. However, the most important receptor on human NK cells specifically mediating TEM across pECs seems to be CD99 [64]. Furthermore, whereas homotypic CD31 interactions are very important for TEM of human leukocytes across human endothelial cells, blocking of CD31 did not influence adhesion or TEM in the pig-to-human combination [32, 64]. This finding agreed with the reported incompatibility between human and porcine CD31 [65]. Finally, one group reported that oxidative stress affects NK cytotoxicity and adhesion to pECs, mainly by reducing the expression of integrins, CD11b, and CD29, on NK cells, and the expression of E-selectin on pECs [66, 67]. Yet, ischemia-reperfusion injury and oxidative stress can be minimised in elective xenotransplantation in contrast to allotransplantation using deceased donor organs which often necessitates cold ischemia during transport of the organ.

In summary, NK cells are recruited to xenografts, perfused organs, or endothelial cell (mono) layers as shown in different models. *In vivo*, NHP NK cells can infiltrate pig organs to a certain degree, whereas ex vivo perfusion and *in vitro* experiments confirmed compatibilities of human and pig adhesion molecules allowing human NK cell recruitment. Molecular incompatibilities on the other hand lead to the activation of both pig endothelium and human NK cells, with consequent proinflammatory chemokine and cytokine production by both cell types. Further *in vivo* investigations using blocking antibodies to key adhesion molecules involved in the recruitment of human and NHP NK cells to pig endothelium, specifically targeting molecules like porcine CD106 (VCAM-1) and human/NHP VLA4 are warranted. In contrast, knocking out pig VCAM-1 to produce transgenic pigs might not work since this approach proved to be lethal in the mouse [68].

3. Recognition and Destruction of Pig Endothelium by Human NK Cells

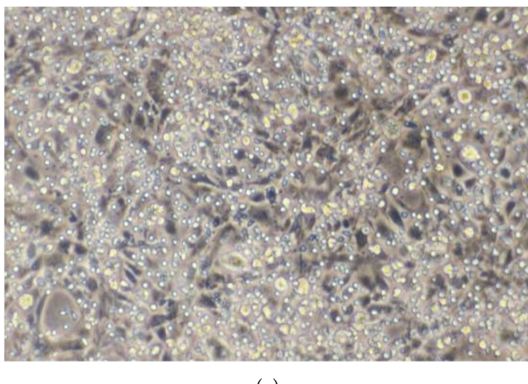
Adhesion of human NK cells to pECs *in vitro* leads to endothelial cell activation and eventually to endothelial cell damage (Figure 1). Malyguine et al. first reported morphological changes on pEC monolayers, the appearance of gaps, and the induction of a procoagulant state by human NK cells [69, 70]. Human NK cells activate pECs in a cell contact-dependent manner, characterized by the induction of E-selectin and IL8 via an NF-κB-dependent pathway; the addition of IgG-containing XenoAbs further enhanced pEC activation and NK cell cytokine secretion (IFNγ and TNF) [71, 72]. Several groups, including our study [73], observed a role of human NK cells in both non-MHC restricted direct cytotoxicity and ADCC against pECs *in vitro*. The majority of these reports was published in the mid-1990s and has been reviewed in detail before [14, 29, 32]. Xenogeneic NK cytotoxicity against pECs can be increased by activation with human IL2, IL12, or IL15, whereas IL8 and IL18 have no effect [74]. The precise role of oligosaccharides, including the αGal epitope, in the direct recognition of pECs by NK cells remains controversial (see Sections 3.1 and 4.1). In addition, human neutrophils recognize pEC independently of natural Abs and C' leading to endothelial activation, associated with increased cell surface expression of VCAM-1 and P-selectin and enhanced NK cytotoxicity [75]; the same might be true for monocytes although it has not been addressed directly. Although the main focus of the present review is on interactions between human NK cells and porcine endothelium, it has also to be mentioned that human NK cells are able to lyse porcine chondrocytes, islets, and embryonic brain-derived cells, via similar mechanisms as described for the destruction of endothelial cells [76–78].

3.1. ADCC against Pig Endothelium Mediated by Xenoreactive Antibodies. Natural or induced XenoAbs deposited on the graft endothelium can be recognized by Fc-receptors (FcRs) on effector cells, including NK cells, causing ADCC. Importantly, deposition of natural XenoAbs of IgG_1 and IgG_2 subclasses occurs on both wild-type (wt) and α1,3-galactosyltransferase knockout (GalT-KO) pECs, whereas IgG_3 deposition was only detected on wt pECs [47]. NK cells express predominantly FcγRIIIa (CD16)

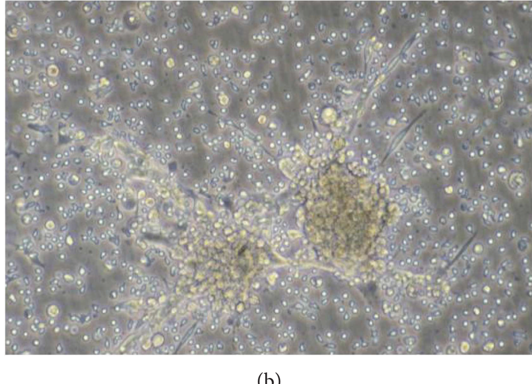
Table 1: Integrins and selectins and their ligands involved in NK cell recruitment to pig endothelium [30, 186].

Protein family	Name	CD name	Heterodimer	*Receptor location	Ligands	Ligand CD name	Ligand location	Cross-species interaction
Integrins	$\alpha 4$	CD49d	$\underline{\alpha 4}/\beta 1$	NK	VCAM-1 fibronectin	CD106 —	pECs ECM	Yes Yes
			$\underline{\alpha 4}/\beta 7$	NK	VCAM-1 MadCAM-1	CD106 —	pEC EC (pEC?)	Yes U
	$\alpha 6$	CD49f	$\underline{\alpha 6}/\beta 1$	NK	Laminin	—	ECM	No
	αL, LFA-1	CD11a	$\underline{\alpha L}/\beta 2$	NK	ICAM-1 ICAM-2	CD54 CD102	pECs pECs	Yes U
	αM, Mac-1	CD11b	$\underline{\alpha M}/\beta 2$	NK	ICAM-3 RAGE	CD50 —	pECs ST	Yes U
	$\beta 1$	CD29	$\alpha 4/\underline{\beta 1}$	See above				
			$\alpha 6/\underline{\beta 1}$	See above				
	$\beta 2$	CD18	$\alpha L/\underline{\beta 2}$	See above				
†Integrin dimers	VLA-4	CD49d/CD29	$\alpha 4/\beta 1$	See above				
	VLA-6	CD49f/CD29	$\alpha 6/\beta 1$	See above				
	LPAM-1	—	$\alpha 4/\beta 7$	See above				
Selectins	E-selectin	CD62E	—	pECs	PSGL-1 sialyl LewisX Sialophoryn	CD162 CD15s CD43	pECs NK NK	No U U
	L-selectin	CD62L	—	NK	Mucosialin MadCAM-1 GlyCAM-1	CD34 — —	pEC pECs	Yes
	P-selectin	CD62P	—	pECs	PSGL-1 Sialyl LewisX	CD162 CD15s	NK NK	No U
	pMIC2?	pCD99?	—	pECs	MIC2, E2	CD99	NK	Yes
Adhesion molecules	PECAM-1	CD31	—	pECs	PECAM-1	CD31	NK	No

*Cell on which the molecule is expressed. †Integrins exist as heterodimers and are composed of one α and one β unit. CD: cluster of differentiation; ECM: extracellular matrix; NK: human natural killer cells; pEC: pig endothelial cells; ST: several tissues; U: unknown.



(a)



(b)

Figure 1: NK cytotoxicity against 2A2 pig endothelial cells. (a) Monolayers of porcine endothelial cells (pEC) were cultured to confluence and a suspension of IL2-activated purified polyclonal NK cells (bright round cells) was added on top of the monolayer, always in the absence of human sera. (b) After 4 hours of coculture with IL2-activated NK cells, the pEC monolayer was destroyed. Pictures were taken with a 200x magnification.

recognizing IgG_1 and IgG_3, and, less efficiently, IgG_2 [79]. ADCC involves the release of the contents of cytotoxic granules and the expression of death-inducing cell surface molecules (FasL, TRAIL) by NK cells. In general, xenogeneic ADCC depends, in addition to the class and subclass of IgG, on the density and stability of the Ag expressed on the surface of the target cell; XenoAbs' affinity for the Ag and FcR-Ab binding affinity. Other innate immune cells including monocytes, macrophages, neutrophils, eosinophils, and dendritic cells can eliminate Ab-coated target cells through phagocytosis and, to a lesser degree, ADCC via their FcR [80].

Human anti-pig ADCC was originally described by Watier et al. in pECs exposed to human peripheral blood mononuclear cells (PBMC) in the presence of human serum, while it could be prevented by the removal of IgG by immune absorption [81]. Moreover, blocking with anti-CD16 Abs abolished ADCC without affecting direct cytotoxicity [81]. Interestingly, no significant cytotoxicity was found in ADCC assays using normal sera or sera from diabetic patients, PBMC as effector cells and porcine islet cells as targets [82]. Moreover, Kumagai-Braesch et al. showed that ADCC was stronger in the presence of purified αGal-specific Abs or anti-pig Abs present in the serum of xenoimmunized patients [76]. Enzymatic removal of αGal from pECs reduced the binding of IgG, most pronounced for IgG_2. However, it did not provide resistance against human IgG-dependent cytotoxicity indicating that αGal was not the only xenogeneic epitope responsible for xenogeneic ADCC [83]. These findings were later confirmed by us using GalT-KO pECs [47].

The generation of human C' regulatory protein transgenic and GalT-KO pigs has largely overcome hyperacute rejection (HAR) in NHP models [3, 84, 85], illustrating the predominant role of αGal. Nevertheless, acute XenoAb-mediated rejection directed against non-αGal Ags still occurred in a pig-to-baboon heart transplantation model [86]. In human serum, some of these non-αGal XenoAbs that induce E-selectin expression on pECs and complement C5b-9 deposition [48] recognize the Hanganutziu-Deicher Ag. This Ag is characterized by a terminal *N*-glycolylneuraminic acid (Neu5Gc) generated by conversion of the activated sugar donor CMP-Neu5Ac into CMP-Neu5Gc by cytidine monophosphate-*N*-acetylneuraminic acid hydroxylase (CMAH) [87–90]. Furthermore, an uncharacterized saccharide on pEC and synthesized by the porcine enzyme beta1,4 *N*-acetylgalactosaminyltransferase (B4GALNT2) is recognized by human XenoAbs [33]. The expression of Neu5Gc and lack of the corresponding natural XenoAbs in NHP, and the fact that deletion of CMAH in GalT-KO pigs increases NHP antibody binding, renders the interpretation of results obtained in pig-to-NHP models difficult [89, 91]. Recently, the non-αGal problem was further addressed by the production of triple-KO pigs lacking the GalT, CMAH, and B4GALNT2 genes in order to abrogate the expression of αGal, Neu5Gc, and the other unknown XenoAg, respectively [91]. Overall, pECs from these triple GalT•CMAHP•B4GALNT2-KO pigs did not support human natural XenoAbs-binding, but ADCC experiments have not been reported so far. Concerning XenoAbs directed against membrane proteins, some anti-HLA antibodies present in the serum of sensitized transplant patients cross-react with SLA class I, which has also been successfully knocked-out in pigs recently [92]. Furthermore, anti-porcine CD9, CD46, CD59, and EC protein C receptor XenoAbs were induced in a pig-to-NHP cardiac xenotransplantation model [93]. In conclusion, genetic modifications have substantially reduced or even eliminated the recognition of pECs by natural XenoAbs. However, the recognition of induced non-αGal XenoAbs by NK cells remains to be addressed in ADCC experiments (Figure 2).

3.2. Receptor-Ligand Interactions Involved in Direct Xenogeneic NK Cytotoxicity against pECs. Freshly isolated, as well as IL2-activated, human NK cells are able to recognize and destroy pECs of different anatomical origin, even in the absence of human XenoAbs indicating that the balance between activating and inhibitory receptors is disrupted. The major ligands recognized by inhibitory NK cell receptors are MHC class I molecules [94]. The human inhibitory killer cell immunoglobulin-like receptors (KIRs), KIR2DL2/2DL3, KIR2DL1, and KIR3DL1, are specific for the HLA-C1,

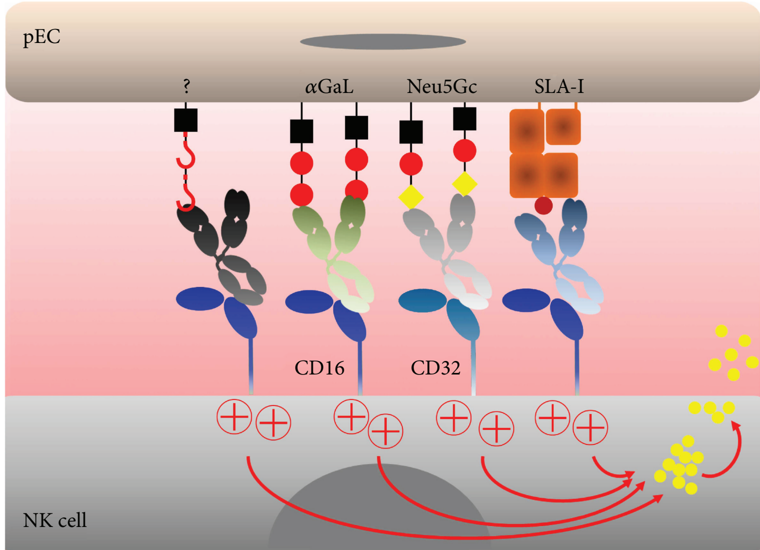


Figure 2: NK cell-mediated destruction of pig endothelial cells by recognition of human anti-pig antibodies (ADCC). Preformed natural XenoAbs circulating in the blood, mainly directed against *α*Gal but also other sugar antigens such as Neu5Gc, bind to pig endothelial cells with their Fab portion. The Fc-fractions of the antibodies are recognized by the FcRs located on the surface of NK cells, for instance, CD16 (Fc*γ*RIIIa) triggering the signaling cascade that leads to NK cell degranulation. The release of their lytic granules containing granzymes and perforin leads to target cell destruction, in this particular context, pig endothelial cells lysis, a process known as antibody-dependent cell-mediated cytotoxicity (ADCC). Alternatively, induced anti-SLA class I antibodies (far right) are recognized by NK cells via CD16, also leading to ADCC. *α*Gal: alpha Gal xenoantigen; HD Ag: Hanganutziu-Deicher antigen; Neu5Gc, SLA-I: swine leukocyte antigen class I.

HLA-C2, and HLA-Bw4 supratypes, respectively [24]. Another important inhibitory receptor on NK cells is immunoglobulin-like transcript 2 (ILT2) that also interacts with MHC class I, both classical and nonclassical [24, 95], and CD94-NKG2A recognizing HLA-E. Amino acid residues critical for the binding to human inhibitory NK cell receptors are altered in SLA class I as compared to HLA class I. Therefore, SLA class I cannot efficiently transmit inhibitory signals to human NK cells [96]. However, this incompatibility may be able to be at least partly overcome in situations where SLA-I expression is increased, such as following pEC activation by TNF or IL1 [59, 97]. Nonetheless, SLA class I molecules seem at least much less efficient compared to HLA class I in inhibiting human NK cells (Figure 3).

Among the known activating NK cell receptors [95], at least three are involved in NK cytotoxicity against pECs, CD2, NKp44, and NKG2D. Early studies showed a reduction of anti-pig NK cytotoxicity by specific blocking of CD2 on IL2-activated PBMC [58]. This effect was attributed to NK cells and not to T cells because blocking of human CD3 had no effect [58]. These initial results were recently confirmed by Kim et al. using purified NK cells and the same blocking strategy with anti-human CD2 Ab. However, the reduction in NK cytotoxicity and production of TNF and IFN*γ* by NK cells were not complete [98]. As to the potential pig ligands of CD2, that is, orthologs of CD58 (LFA-3) and CD59, blocking with anti-pig CD58 efficiently inhibited lysis of porcine targets by human PBMC to the same extent as anti-CD2 [98, 99]. Blocking of the adhesion molecule LFA-1 (CD11a/CD18) as well as of CD16, CD8, and CD57 on NK cells did not inhibit NK cytotoxicity against pECs [58] (Figure 3).

A role of NKp44 and NKG2D was demonstrated by reversal of NK cytotoxicity against pECs in the presence of blocking Ab, whereas other NK receptors including NKp30, NKp46, 2B4, CD49d, and CD48 were not involved [98, 100]. Of note, complete protection of pECs against cytotoxicity was only achieved when combinations of anti-NKp44 and -NKG2D or anti-CD2 and -NKG2D blocking Abs were used [98, 100]. As to the potential pig NKG2D ligands, we identified the ortholog of ULBP-1 (pULBP-1) and showed that it bound to human NKG2D, whereas pMIC2, another NKG2D ligand, was not involved in pEC destruction [101]. Interestingly, human serum-induced pULBP-1 on pECs, whereas treatment with either pig or human TNF or human cytomegalovirus infection of pECs led to a reduction of its expression [102]. Subsequently, Tran et al. detected an additional ligand of human NKG2D in porcine cells, the precise nature of which still remains unknown [103] (Figure 3).

The contribution of the costimulatory pathway CD28-CD80/CD86 to NK cytotoxicity against pECs has also been analyzed. A variant form of CD28 (vCD28) is expressed in subpopulations of NK cells. On the other hand, porcine cells including pECs and fibroblasts express pCD80/CD86, both constitutively and following exposure to T and NK cells [104, 105]. Blocking with a species-specific anti-pig CD86 antibody reduced xenogeneic NK cytotoxicity, whereas blocking pECs with anti-human CD80, CD86, and CD154 did not show any effect [105], indicating that interactions between vCD28-pCD86 are preserved across the species barrier [106]. In addition, blocking the vCD28-pCD86 pathway delayed xenograft rejection by inhibiting T and NK cell activation in a small animal cell transplantation model [107].

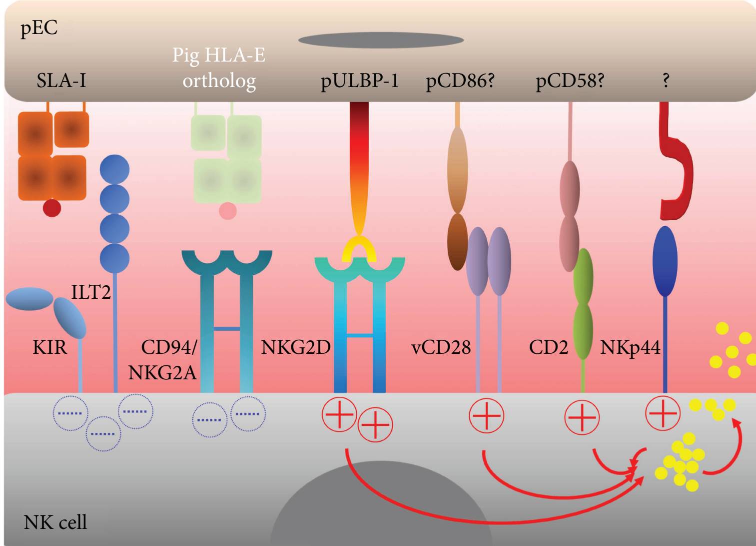


Figure 3: Receptors and ligands involved in pig endothelial cells lysis by human NK cells. There is a tight balance between activating and inhibitory signals that control NK cytotoxicity. The activating NK receptors NKG2D and NKp44 bind to their pig ligands: pULBP-1 and an unidentified molecule, respectively, and trigger lytic granule release (shown by red arrows and yellow circles). A role of CD2 and variant CD28 in facilitating NK cytotoxicity has been described in NK subpopulations, potentially by interacting with porcine CD58 and CD86, respectively. The inhibitory NK receptors, KIR, ILT2, and CD94/NKG2A, poorly recognize porcine MHC-I molecule (SLA-I) including the pig ortholog for HLA-E leading to a lack of inhibitory signals (in dotted blue) and NK cell activation.

However, vCD28 has not been directly tested in the pig-to-human xenogeneic context (Figure 3).

3.3. Effector Mechanisms of Xenogeneic NK Responses. NK cytotoxicity is characterized by the pH-dependent release of perforin from lytic granules. Consequently, perforin assembly leads to pore formation in the target cell membrane and necrotic cell death. Moreover, these channels also enable other granule components including granzymes and granulysins to enter and to induce caspase activity and apoptotic cell death. Alternatively, apoptosis of target cells can be initiated by death receptor pathways including interactions between FasL expressed on NK cells and Fas on target cells. The latter mechanism does not play a role in xenogeneic NK cytotoxicity, because cross-species signaling between human FasL on NK cells and porcine Fas was only demonstrated using transfected porcine PK15 targets cells overexpressing pig Fas [108]. In contrast, a role of perforin and granzymes in human anti-pig NK cytotoxicity was demonstrated *in vitro* by the group of Nakajima and our own group using compounds that disturb the acidification of lytic granules (concanamycin A/B and ammonium chloride) and Ca^{2+} chelators that inhibit perforin polymerization [109–111]. A pan-caspase inhibitor prevented the lysis of pECs by human NK cells only partially in the absence of human serum [110], whereas specific caspase inhibitors demonstrated that only caspase-3 and -8, but caspase-1, are involved in ADCC mediated by XenoAbs [112]. Taken together, perforin/granzyme-dependent apoptosis and osmolysis, but not the FasL death receptor pathways, are implicated in human NK cytotoxicity against pEC. Finally, NK cells, beyond their function as cytotoxic effector cells, may initiate and regulate adaptive immune responses, thereby promoting either rejection or tolerance, as shown in solid organ allotransplantation [10–12]. Very little is so far known in the field of xenotransplantation. One study reported that NK cells eliminate cellular xenografts in a pig-to-mouse model via IFNγ but independently of perforin [113]. Another study showed that marginal zone B cells need help from NK cells to produce XenoAbs, a process that is independent of T cells and neither requires cytotoxicity nor IFNγ production [114].

In conclusion, the large majority of our knowledge on the mechanisms leading to human NK cell-mediated porcine endothelial cell recognition and destruction was generated *in vitro*. The role of NK cytotoxicity in pig-to-NHP xenograft rejection remains to be addressed more closely. It is likely, although not yet experimentally proven, that the immunosuppressive protocols currently used also inhibit NK cell functions (see below). However, as shown in some *in vivo* models, NK cells are also involved in promoting acquired xenoresponses or destruction of cellular xenograft mediated by cytokine production. It would be therefore of interest to study the effect of long-term NK cell depletion in preclinical *in vivo* models.

4. Strategies to Protect the Porcine Endothelium from NK Cytotoxicity

As expected, once that the major molecules involved in the interactions between human cells and pECs were characterized, the next step was to investigate whether it is feasible to manipulate these interactions or to identify inhibitory mechanisms to reduce the activity of human NK cells against pECs. A broad summary of successful strategies

Table 2: Proven strategies to overcome NK cytotoxicity against pig endothelial cells.

Target	Approach	NK source	Effect	Reduction (%)	Ref
αGal GT	*In vitro* knockout pEC lines	IL2-NK cells, NK92 Fresh NK cells, Fresh NK cells	No effect CMC ↓ ADCC ↓ nAb/CML	NA 77–90 86	[46, 126]
	Gal knockout pigs	IL2-NK cells, Fresh NK cells Fresh NK cells	No effect in CMC ↓ ADCC ↓ nAb/CML	NA 70 80	[47, 124]
	siRNA in pEC	NK92 cell line	No effect CMC ↓ ADCC	NA 30–40	[125]
Masking of sugar xenoantigens	Transfections of α(1,2)-fucosyltransferase in pECs	Fresh NK cells	↓ CMC	47	[119]
	Treatment of pECs with DXS	NK92	↓ CMC inh. C' deposition	25–47	[135]
HLA class I molecules	HLA-E transfection in pEC	IL2-NK cells	↓ CMC	15–60	[150, 151]
	HLA-E transgenic pigs	IL2-NK cells	↓ CMC ↓ IFNγ production	8–30 40	[15]
	HLA-G1 transfection in pEC	IL2-NK cells, NK92 and NK cell clones	↓ CMC ↓ rolling/adhesion No effect in ADCC	20–45 25–75 NA	[141, 142, 150, 187]
	Soluble HLA-G1	NK92 Fresh PBMC	↓ CMC ↓ CMC	31–83 24	[145]
	HLA-Cw3 transfection in pEC	NK cell clones	↓ CMC	12–70	[136]
	HLA-Cw4 transfection in pEC	NK cell clones	↓ CMC	58	[139]
	HLA-B27 transfection in pEC	NK cell clones	↓ CMC	~30	[136]
Apoptosis induction	PK15, human FasL transfection	PBL	↓ ADCC	34–42	[108]
	PK15, human FasL transfection	PBL, LAK	↓ ADCC ↓ CMC	54 74	[127]
	pEC, human FasL transfection	IL2-NK cells	No effect CMC ↑ apoptosis NK cells	0	[129]
	pEC, pig FasL transfection	IL2-NK cells	↓ CMC ↓ FasL apoptosis	26 23	[128]
Apoptosis resistance	Bcl-2 transfection in PK15	PBL	↓ FasL apoptosis ↓ ADCC	62 50	[132]
	Bcl-2^{mut} transfections in pEC	IL2-NK cells	No effect CMC	0	[110]

ADCC: antibody-dependent cell-mediated cytotoxicity; αGal GT: alpha1,3-galactosyltransferase; C': complement; CML: complement-mediated lysis, CMC: cell-mediated cytotoxicity; DXS: dextran sulfate; HLA: human leukocyte antigen; IFNγ: interferon gamma; inh.: inhibition; LAK: lymphokine-activated killer; NA: not applicable; nAb: natural antibody; PBL: peripheral blood lymphocytes; PBMC: peripheral blood mononuclear cells; pEC: porcine endothelial cells; PK15: pig kidney cell line; siRNA: short interfering RNA; ↓: reduction.

shown to reduce these interactions is summarized in Table 2. Despite the identification of the major adhesion molecule interactions responsible for NK cell binding to pECs and the availability of commercialized monoclonal Abs blocking, for example, VLA4 (natalizumab), there are essentially no studies trying to test adhesion blocking approaches *in vivo* or ex vivo [14, 115]. Thus, we will pay special attention to approaches to control human anti-pig NK cytotoxicity: (i) the potential of carbohydrate modifications such as αGal knockout to prevent NK cell responses; (ii) manipulations of apoptotic pathways and dextran sulfate; and (iii) expression of human MHC class I molecules on pECs binding to inhibitory human NK cell receptors that initiate immunoreceptor tyrosine-based inhibitory motif-(ITIM0-) dependent negative signaling pathways [116].

4.1. Removal of αGal Epitopes and Modification of Other Sugar Antigens. The removal of αGal from pigs has been a great advance in the field of xenotransplantation as HAR was avoided in pig-to-baboon xenotransplantation models [117, 118]. The contribution of αGal to NK cell-mediated responses in the absence of natural or induced anti-αGal Abs remains controversial. Some groups reported that human NK cells directly recognize oligosaccharide ligands expressed by xenogeneic cells [87, 106, 119, 120]; carbohydrate remodeling of pECs, for instance, increased the

susceptibility to human NK-mediated lysis as demonstrated by transfection of $\alpha(1,2)$-fucosyltransferase in pECs [119]. Christiansen et al. reported interactions between the human NK receptor NKRP1A and αGal [121]. In contrast, we and others could not confirm the role of αGal as a dominant cytotoxicity-inducing NK target molecule when testing NK cytotoxicity against GalT-KO or GalT RNA-silenced pEC [46, 122–126]. A significant difference between these latter and earlier experiments was that there was no need to treat the target cells with either galactosidase or blocking reagents [46, 122–126]. NK cell-mediated ADCC was reduced by 30 to 70% in the absence of αGal expression on pECs, whereas direct xenogeneic lysis mediated either by freshly isolated or IL2-activated human NK cells or the NK cell line NK92 was not reduced [46, 124]. Nonetheless, full elimination of αGal from pECs prevented complement-induced lysis (up to 86%) and ADCC (from 30–70%) but not direct xenogeneic human NK cytotoxicity mediated by freshly isolated, IL2-activated NK cells or the NK-92 cell line [46]. Conversely, NK cell-pEC adhesive interactions were not reduced [46]. In addition, αGal-independent interactions between human NK cells and pECs triggered an intracellular Ca^{2+} rise in pECs, followed by an upregulation of P-selectin and VCAM-1, and NK cell activation resulting in increased expression of perforin and cytotoxicity [122]. Transgenic expression of αGal on primary human aortic endothelial cells, as shown by He's group did neither trigger NK cytotoxicity nor adhesion [123]. However, these results do not completely rule out a role for αGal in NK recognition; it may be necessary but not sufficient to interfere with αGal to overcome xenogeneic NK responses. The recent generation of KO pigs for multiple saccharide XenoAgs resulted in no remaining binding of human Abs [91], but the direct effect on NK cell responses has not been tested yet.

4.2. Manipulation of Apoptotic Pathways. Two approaches to protect pig endothelial cells from NK cytotoxicity by genetic engineering of the apoptotic pathways have been explored: (i) overexpression of FasL in order to induce lysis of activated Fas-expressing human effector cells including NK cells; and (ii) overexpression of antiapoptotic proteins such as Bcl-2 and A20 to counter-balance proapoptotic signals induced by NK cells.

Transfection of pig epithelial cells (PK15) with human FasL provided partial protection against human NK cytotoxicity [108, 127]. In addition, overexpression of porcine FasL reduced the susceptibility to lysis by IL2-activated human NK and T cells by inducing apoptosis [128]. In our hands, expression of human FasL on pECs did not provide protection against human NK cytotoxicity, although apoptosis of human NK cells was observed. Moreover, human FasL expression had no effect on NK cell adhesion to pECs. In contrast, NK cell migration through pECs and chemotaxis of human polymorphonuclear cells were strongly increased by the expression and cleavage of soluble FasL [129], consistent with earlier reports [130].

Late in the ‘90s, considerable levels of the antiapoptotic proteins Bcl-2 and A20 were found in the graft endothelial cells in rodent xenografts that were “accommodated” [131]. Overexpression of Bcl-2 in pEC did not provide protection against human NK cytotoxicity in our hands [110]. In contrast, in another preliminary study by Nakajima et al. Bcl-2 expression in PK15 cells provided partial protection against apoptosis caused by human perforin/granzyme in ADCC assays or Fas/FasL interactions [112, 132]. Finally, transgenic pigs expressing A20 on their endothelial cells have been generated, but the protective role of A20 against human NK cytotoxicity was formally not yet studied [133, 134].

4.3. Dextran Sulfate Protects Pig Endothelial Cells from NK Cell-Mediated Cytotoxicity. Another approach to protect pECs from NK cytotoxicity explored the use of low molecular weight dextran sulfate, an analog of proteoglycans that are shed from pECs upon activation. Indeed, there was a protective effect when pECs, activated or not with pig TNF, were exposed to human NK cells in the presence of dextran sulfate. This protection was specific for pECs because when the prototypical NK target cell K562 was preincubated with dextran sulfate, the NK cytotoxicity was not affected [135].

4.4. Expression of Classical HLA Class I Molecules HLA-Cw4 and HLA-Cw3 in Porcine Endothelial Cells Inhibits NK Cytotoxicity. Certain HLA class I supratypes bind to inhibitory killer cell immunoglobulin-like receptors (KIRs), for example, CD158a (KIR2DL1) and CD158b (KIR2DL2/3). Specific natural ligands for CD158a and CD158b are HLA-Cw4 and HLA-Cw3, respectively [24]. Transfection of pECs with plasmids encoding HLA-Cw4 and HLA-Cw3 led to partial protection from bulk human NK cytotoxicity and complete protection from NK clones with high expression of CD158a [136]. Taking this approach one step further, Sharland et al. reported that a porcine B-lymphoblastoid cell line transfected with HLA-Cw*0304 gene constructs encoding genetically modified HLA-Cw3 unable to interact with CD8, inhibited both direct cytotoxicity and ADCC mediated by human NK clones expressing the appropriate CD158b inhibitory receptor while avoiding recognition by human $CD8^{+}$ T cells [137, 138]. However, expression of both HLA-Cw3 and -Cw4 did not confer further protection. Intriguingly, the expression of HLA-Cw4 also reduced the adhesion of human NK cells to pECs [136, 139]. Finally, expression of HLA-B27 on pECs provided only moderate to low protection from NK lysis even when NK cells derived from HLA-B27 positive donors were tested. HLA-A2 expression did not protect from xenogeneic NK cytotoxicity [136].

4.5. Expression of HLA-G in Porcine Endothelium Inhibits NK Cytotoxicity and Adhesion. HLA-G is a nonclassical human MHC class I molecule with limited polymorphism compared to classical HLA class I alleles. In addition, HLA-G inhibits human NK cytotoxicity without inducing T cell alloresponses. The human ligands for HLA-G are KIR2DL4 (CD158d) and immunoglobulin-like transcripts 2 and 4 (ILT2, ILT4). These are expressed at different levels on NK cells and other cells and therefore made this molecule attractive to study in the context of pig-to-human xenotransplantation [140]. Initial work showed that transfection of pECs with HLA-G only had a modest protective effect on NK

cytotoxicity and that the expression of ILT-2/LIR-1 on NK cells did not correlate with the HLA-G mediated inhibition [141–143]. Surprisingly, HLA-G reduced rolling adhesion of activated human NK cells on pECs adding a new function to this nonclassical HLA class I molecule [144]. Finally, the group of Chen et al. showed that pECs were protected from human NK cytotoxicity by soluble HLA-G_1 [145]. In contrast to HLA-E (Section 4.6.), HLA-G protects porcine cells from lysis by human NK cells through a CD94/NKG2-independent pathway [142, 146, 147].

4.6. Expression of HLA-E in Porcine Endothelium Inhibits Xenogeneic NK Cytotoxicity. HLA-E is another nonclassical HLA class I molecule restricted to only two functional variants making it attractive to use in xenotransplantation. HLA-E is recognized by the inhibitory NK cell receptor CD92/NKG2A and by the activating receptor CD92/NKG2C, although with 5–10 fold lower affinity [140, 148]. In fact, HLA-E provided partial protection of transfected pECs from polyclonal xenoreactive human NK cell populations and total protection when NK clones expressing high levels of NKG2A were used as effectors [149, 150]. In contrast to what was observed for HLAC-Cw4 and HLA-G, HLA-E had no effect on the adhesion of human NK cells to pECs [151], a result which was recently confirmed in an elegant *in vitro* flow system under dynamic conditions using pEC stemming from HLA-E transgenic pigs [20].

These *in vitro* results, in combination with the fact that HLA-E is relatively nonpolymorphic and thus of low alloreactivity, stimulated the generation of double HLA-E/human β2microglobulin transgenic pigs in a collaborative project with the Munich group [15]. Indeed, pECs derived from these transgenic animals showed partial protection against human NK cytotoxicity, depending on the level of expression of CD94/NKG2A on NK cells, and lower production of IFNγ by NK cells in response to pECs [15].

In order to take these studies a step further, several ex vivo xenoperfusion models have been established allowing investigations on the interactions between human blood and pig tissues directly. Collaborating with a multidisciplinary team in Berne, we explored a pig limb perfusion system using human blood [16]. Compared to wild-type pig limbs, humoral xenoresponses were reduced in double transgenic pigs expressing human CD46, a C' regulatory protein, and HLA-E. Moreover, NK cells were quickly removed from the circulating blood infiltrating the muscle tissue. Slightly delayed NK cell recruitment and reduced tissue infiltration were observed in perfused HLA-E/hCD46 double transgenic pig limbs [18]. The expression of HLA-E in transgenic pigs has also been tested in ex vivo lung and heart perfusion with human blood, and showing similar results in terms of reduced tissue damage, most likely linked to a reduction of NK responses [19, 20]. Since the expression of the HLA-E receptor CD94/NKG2A on NHP NK cells and functional inhibition of NHP NK cells by HLA-E remains to be addressed experimentally (Section 6), it is hard to judge whether currently used pig-to-NHP models are appropriate to test the role of HLA-E in inhibiting human anti-pig xenogeneic NK cell responses *in vivo*.

4.7. Effect of Immunosuppressive Drugs and Biologicals on NK Cells. The mechanisms of T and B cell inhibition mediated by conventional immunosuppressive drugs (ISD) and biologicals used in transplantation medicine are well known. As to NK cells, the literature reports a variety of specific effects of ISD on NK cells with to some extent conflicting conclusions [152–160]. A comprehensive comparative study confirmed that corticosteroids are potent inhibitors of NK functions including ADCC, direct NK cytotoxicity, and IFNγ production (own unpublished data). In addition, NK cytotoxicity was inhibited by the highest therapeutic doses of cell cycle (mycophenolate mofetil) and mTOR inhibitors (everolimus). As to calcineurin inhibitors, cyclosporine inhibited direct cytotoxicity, whereas tacrolimus reduced both, ADCC and direct cytotoxicity. Little is known so far on the effect of biologicals or monoclonal antibodies on NK cell function, for example, tocilizumab (anti-IL6R), infliximab (anti-TNF), and natalizumab (anti-VLA4) [161]. Moreover, monoclonal antibodies that block costimulation such as anti-CD154 and anti-CD40 are being used in xenotransplantation models with great success [162], but their effect on NK cells is largely unknown. Expression of CD154 on NK cells upon IL2 stimulation increased NK cytotoxicity in one study [163], and human NK cells were shown to activate autologous human B cells via CD40-CD154 interactions [164]. On the other hand, treatment with an agonistic anti-CD40 reduced NK cell numbers in the circulation in one cancer study [165], but nothing has been published in the transplantation field. Taken together, it is very likely that the immunosuppressive protocols currently used in preclinical pig-to-NHP xenotransplantation models have an important inhibiting impact on NHP NK cells but this remains to be addressed in more detail in future studies.

As outlined in this chapter, several different strategies have been developed to control NK cell xenoresponses. In addition to the removal of αGal and other oligosaccharide ligands of preformed anti-pig XenoAbs leading to the reduction of ADCC, the generation of HLA-E transgenic pigs is the most promising approach, although there is still not enough *in vivo* evidence to fully support the relevance of this strategy to protect pig xenografts from human NK cell-mediated injury. Finally, ISD, in particular corticosteroids, inhibit NK cells, but the effect of ISD, especially the new generation of biologicals on NHP/human NK cells, warrants further exploration. However, the "Holy Grail" would be to find ways to induce NK cell tolerance towards xenografts and to avoid ISD and their side effects.

5. Modulation of Xenogeneic NK Responses by Other Immune Cells

Emerging strategies to control "unwanted" NK cell-mediated immune responses in xenotransplantation not only include inhibition of NK cells or manipulation of their presumed targets (specifically pEC) by genetic engineering of cell surface molecule expression patterns but also the use of regulatory immune cells of recipient origin to induce transplantation tolerance.

One important approach to induce transplantation tolerance is the mixed hematopoietic chimerism model. The group of Sykes discovered that NK cells become specifically tolerant to donor cells in murine mixed allogeneic chimeras, whereas NK cell tolerance was associated with global unresponsiveness of the murine NK cells in the xenogeneic rat-to-mouse mixed chimerism model [166]. More recently, the same group studied the effect of mixed porcine chimerism on human NK cell phenotype and function, xenogeneic cytotoxicity, and IFNγ production, in a humanized mouse model with induced NK cell reconstitution. Interestingly, variable and partial human xenogeneic NK cell tolerance to pig cells was demonstrated in cytotoxicity assays [167]. The effect of this promising tolerance approach remains to be further explored and tested on NK cells in NHP models.

Whereas the various cell populations involved in transplant rejection and strategies on how to suppress them are well known, the cell populations involved in the induction and maintenance of transplant tolerance remain less well characterized. In particular, the respective and precise contribution of DC and T cells in cell-mediated xenograft rejection and tolerance induction is not yet fully dissected. Human DC effectively adhered to pEC and were activated by xenoantigens, resulting in highly efficient antigen presentation and proliferation of $CD4^+$ T cells [168]. On the other hand, porcine + derived from huTRAIL transgenic pigs decreased human T cell proliferation significantly without any signs of apoptosis [169]. *In vitro* human $CD4^+CD25^+$ regulatory T cells suppressed indirect xenogeneic immune responses mediated by DC pulsed with porcine epithelial cells [170]. Moreover, human myeloid-derived suppressor cells were shown to inhibit anti-pig xenogeneic responses mediated by human NK cells and cytotoxic T lymphocytes (CTL) [171]. Finally, Ierino's group demonstrated that the use of pig DC with tolerogenic properties significantly reduced human T cell responses when used as stimulators in human lymphocyte proliferation assays *in vitro* [172].

Recently, we reported the efficiency of two different human monocyte-derived DC generated under tolerogenic conditions, that is, in the presence of IL10 and rapamycin, to control xenogeneic NK cells and CTL. Indeed, IL10-DC were able, at least *in vitro*, to decrease NK degranulation and intracellular IFNγ production in response to pEC. In addition, tolerogenic IL10-DC reduced xenogeneic CTL cytotoxicity in a haplotype-specific manner [173]. *In vivo*, mouse IL10-DC were used in a concordant rat-to-mouse islet xenotransplantation model, showing an overall increase of xenograft survival, the mechanisms of which are currently under investigation [174]. Furthermore, human regulatory T cells (Treg) have been shown to inhibit NK cells by several different mechanisms [175] and also to suppress xenogeneic immune responses [176]. Recruitment of human Treg to pECs depends on particular chemokine receptors (CXCR3 and CCR4) and integrins (CD18 and CD49d). *In vitro*, human Treg partially suppressed xenogeneic human NK cell adhesion to pECs, as well as xenogeneic cytotoxicity and degranulation [177]. Taken together, these results will help to develop new protocols to specifically regulate NK cell-mediated xenograft responses by using Treg and tolerogenic DC.

In conclusion, modulation of cellular immune responses by mixed chimerism induction and cell therapy has been tested successfully in both allotransplantation and xenotransplantation models. The mixed chimerism approach has even been successfully used in clinical trials but is still limited by the inherent toxicity of hematopoietic stem cell transplantation in NHP and humans. Strategies using Treg and tolerogenic DC to prevent graft rejection are attractive new frontiers that are now being translated to the clinic and to the field of xenotransplantation research.

6. The Role of NK Cells in Preclinical Nonhuman Primate Models

Whereas the molecular interactions between human NK cells and pig cells can be rather easily studied *in vitro*, it is much more difficult to draw firm conclusions on their relevance *in vivo*. Though very helpful for hypothesis-driven basic research, rodent models are often of limited value for human conditions. The translation of results stemming from these experiments to human clinical application remains largely elusive due to fundamental differences of the respective immune systems. While humanized mouse models and restoring physiological microbial exposure in mouse husbandry may provide some improvement [178, 179], progress in xenotransplantation research relies essentially on NHP models. Nevertheless, differences in NK cell biology between humans and baboons or cynomolgus monkeys should be taken into consideration when analyzing immune responses in pig-to-NHP xenografts. Early immunohistological studies have suggested that NK cells are involved in pig-to-NHP xenograft rejection, but these analyses suffered from unreliable staining techniques [38, 40]. *In vitro*, a number of groups have analyzed the phenotype, natural cytotoxicity, and antiporcine function of NHP NK cells [38, 40, 180–182]. Destruction of pig endothelium mediated by baboon NK cells via ADCC and direct cytotoxicity and spontaneously enhanced following IL2-activation was demonstrated and depended on CD2 and CD49d as shown by antibody blocking studies [40]. Furthermore, peripheral blood lymphocytes (PBL) from nonsensitized baboons spontaneously lysed pEC, which was inhibited by both anti-CD2 and anti-CD94 blocking. Reduction of galactosyl residues by galactosidase digestion decreased almost completely pEC lysis by nonsensitized, but not primed, baboon PBL [181]. Finally, baboon NK cells can be identified and isolated on the basis of a $CD3^-NKp46^+CD8^{dim}CD16^{+/-}$ or $CD3^-CD8^{dim/-}CD16^{bright}$ phenotype and expressed CD56 upon IL2 activation. They exert very low spontaneous cytotoxicity against both human (K562) and pig target cells, but it can be significantly stimulated by IL2 activation [182].

Little is known as to the expression of other receptors on baboon NK cells including NKp30, NKp44, NKG2A, NKG2D, and KIR/CD158, while expression of KIR, CD94/NKG2A, and NKp80 has been demonstrated on rhesus monkey NK cells [183–185]. However, validation of cross-species reactivity of monoclonal rodent-anti-human antibodies used

to phenotype NHP NK cells remains a problem. In summary, a better molecular and functional characterization of NHP NK cells is warranted for studying their role in porcine xenograft rejection using preclinical pig-to-NHP models.

7. Conclusions

Big advances characterize the past 25 years of xenotransplantation research and in particular the elucidation of the role of NK cells. Although the evidence for a role of NK cells in preclinical pig-to-NHP xenotransplantation models is still weak, it became clear that NK cell anti-pig responses most likely do play a role in xenograft rejection, especially early in the process. They are also involved in shaping xenoresponses mediated by the adaptive immune system. The molecular cross-talk between human NK cells and porcine endothelium that governs recognition and activation of NK cells as well as induction of ADCC via XenoAbs and direct perforin/granzyme-dependent cytotoxicity has been elucidated. Many of these interactions can be explained by either molecular cross-species incompatibilities or intact receptor-ligand interactions between pig ECs and human NK cells. The efforts to understand and to overcome human anti-pig NK cell responses have led to the generation of HLA-E transgenic pigs, which provide at least partial protection, and the advent of new strategies aiming at tolerizing NK cells. In our view, future studies on the NK cell biology of NHP are needed to further elucidate the role of NK cells in pig-to-NHP xenotransplantation preclinical models. To perform these studies and to go beyond the histological characterization of xenograft infiltration, species-specific tools such as antibodies and NK functional assays need to be established. Additionally, it would be of interest to explore *in vivo* the role of new ISD like costimulation blockers in NHP xenotransplantation models to prevent NK cell-mediated xenograft rejection and to induce graft tolerance.

Abbreviations

Ab: Antibody
ADCC: Antibody-dependent cell-mediated cytotoxicity
Ag: Antigen
αGal: Alpha Gal xenoantigen
C': Complement
FcγR: Fc-gamma receptor
FasL: Fas ligand
GalT-KO: Knockout for the enzyme α1,3-galatosyltrans ferase
Grz: Granzyme
HAR: Hyperacute rejection
HLA: Human leukocyte antigen
IFNγ: Interferon gamma
KIR: Killer cell immunoglobulin-like receptor
KO: Knockout
MIC: MHC class I chain related protein
NK: Natural killer
NHP: Nonhuman primates
PBMC: Peripheral blood mononuclear cells
PBL: Peripheral blood lymphocytes
pECs: Porcine endothelial cells
SLA class I: Swine leukocyte antigen class I
TEM: Transendothelial migration
TNF: Tumor necrosis factor alpha
TRAIL: TNF-related apoptosis-inducing ligand
ULBP: UL-binding protein
VLA: Very late antigen
wt: Wild-type
XenoAbs: Xenoreactive antibodies.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

Porcine to Human Heart Transplantation: Is Clinical Application Now Appropriate?

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Cardiac xenotransplantation (CXTx) is a promising solution to the chronic shortage of donor hearts. Recent advancements in immune suppression have greatly improved the survival of heterotopic CXTx, now extended beyond 2 years, and life-supporting kidney XTx. Advances in donor genetic modification (B4GALNT2 and CMAH mutations) with proven Gal-deficient donors expressing human complement regulatory protein(s) have also accelerated, reducing donor pig organ antigenicity. These advances can now be combined and tested in life-supporting orthotopic preclinical studies in nonhuman primates and immunologically appropriate models confirming their efficacy and safety for a clinical CXTx program. Preclinical studies should also allow for organ rejection to develop xenospecific assays and therapies to reverse rejection. The complexity of future clinical CXTx presents a substantial and unique set of regulatory challenges which must be addressed to avoid delay; however, dependent on these prospective life-supporting preclinical studies in NHPs, it appears that the scientific path forward is well defined and the era of clinical CXTx is approaching.

1. Introduction

About 5.7 million Americans have heart failure, half of whom will die within 5 years [1]. Organ transplantation is currently the preferred solution for treatment of end-stage heart failure but less than 3000 heart transplants have been performed annually in the US in recent years. Circulatory assist devices and total artificial hearts have been approved to support patients in chronic heart failure [2, 3]. These mechanical solutions are effective, at least in the short term, but have significant morbidity from thromboembolism, infection, gastrointestinal bleeding, and reduced quality of life [4]. Regenerative solutions for heart failure remain a nascent experimental technology. Cardiac xenotransplantation (CXTx) is a promising viable near-term solution to the shortage of hearts for clinical transplantation. In recent years, there has been a remarkable improvement in survival of heterotopic pig-to-nonhuman primate (NHP) CXTx [5–8], encouraging early success in orthotopic CXTx (oCXTx) [9–11] and advances in life-supporting renal xenotransplantation (RXTx) [12, 13]. These results validate the physiological compatibility of porcine organs, at least in NHPs, and suggest that clinical CXTx may soon be applicable if oCXTx can attain similar improvements in survival as RXTx. In this review, we examine developments in immune suppression, porcine donor genetics, preclinical transplants, and infectious disease issues and discuss requirements for clinical CXTx.

To justify a clinical xenotransplantation (XTx) program, it is necessary to demonstrate transplant efficacy in clinically relevant animal models. The International Society of Heart Lung Transplantation (ISHLT) has suggested that a prospectively defined series of life-supporting cardiac xenotransplants in NHPs, using predefined immune suppression, with "60% survival at 3 months with a minimum of 10 animals surviving for this period," would be sufficient to consider a clinical trial [14]. The recent survival achieved after heterotopic CXTx (hCXTx), in excess of 2 years and with a median survival of 298 days [8], suggests that this goal may be attainable. While the ISHLT recommendation has become a de facto guideline for researchers in

Table 1: The longest (median) reported heterotopic cardiac xenograft survival as a function of donor genetics and immune suppression.

Donor	Earlier immune suppression		Costimulation blockade	
	CsA/CyP/steroid	ATG/CD20/tacrolimus/sirolimus	ATG/LoCD2b/CVF/anti-CD154/MMF	ATG/anti-CD40, CD20/CVF/MMF
WT	$32^{§}$ (21 d) [27] $25^{‡}$ (12 d) [28]	n.r.	n.r.	n.r.
WT;hCRP	99^{Δ} (26 d) [29] $78^{*\Delta}$ (35 d) [30]	$109^{*\#†}$ (20 d) [31] $137^{*\#†}$ (96 d) [32]	139^{*} (27 d) [36]	n.r.
GTKO	n.r.	$128^{†}$ (22 d) [34]	179 (78 d) [37]	n.r.
GTKO;hCRP	n.r.	$52^{\nabla†}$ (28 d) [34]	$8^{¶}$ (8 d) $236^{†¶}$ (71 d) [5]	$149^{¶}$ (84 d) [25]
GTKO;hCRP;TBM	n.r.	n.r.	n.r.	$945^{¶}$ (298 d) [8]

n.r.: none reported. §Soluble CR1 to block complement activation. ‡Cobra venom factor at 0.25–0.5 mg/kg prior to surgery and 0.1–0.5 mg/kg every 1–4 days thereafter. *Included use of alpha-Gal polymer GAS914 [127] or Nex1285 [128]. †Immune suppression included anti-CD20 (Rituximab) B-cell depletion. $^{\Delta}$hDAF (human CD55) minigene [129]. $^{\nabla}$A murine H-2Kb regulated human CD55 transgene [77]. $^{\#}$hCD46 transgene based on 60 kb human genomic CD46 DNA [130]. ¶hCD46 transgene based on a human CD46 minigene [131].

the field, the FDA, responsible for regulating XTx, has not officially endorsed these specific criteria, making early interactions with regulators essential to advance a clinical study.

The most prominent variables which contribute to XTx efficacy are likely to be immune suppression and donor genetics, but recipient species [15], viral status [16–18], the level of preexisting anti-pig antibody [13], prophylactic antiviral and antibacterial therapy [19, 20], and postoperative care [21] also significantly contribute to graft survival. The relative contribution of immune suppression and donor genetics is incompletely understood in a field undergoing rapidly evolving experimental changes in both components. Past reviews of the state-of-the art in XTx [22–24] and recent publications [8, 25] are useful for gaining an appreciation for the breadth of changes in XTx organ survival over the last 20 years. Table 1, showing the longest reported (and median) graft survival for various donor genetics under four broadly defined immune suppression regimens, summarizes these advances for CXTx. Not surprisingly, all combinations of donor genetics and immune suppression have not been reported, as perceived advances in donor genetics were seldom tested with reduced or previous immune suppression techniques.

2. Earlier Immune Suppression

Early hCXTx studies using Gal-positive (WT) donor hearts focused on preventing hyperacute rejection and often used immune suppression based largely on cyclophosphamide, cyclosporine, and steroids (CCS) [26]. Maximal hCXTx survival of 32 days was achieved in pig-to-cynomolgus monkey transplants using soluble CR1 to block systemic complement activation [27]. Comparable results were also reported after pig-to-baboon hCXTx using cobra venom factor (CVF) to consume complement [28]. CCS immune suppression was also used in early hCXTx studies using transgenic donors expressing human complement regulatory proteins (hCRPs). The longest reported survival was 99 days (median 26 days) using hDAF transgenic hearts [29]. These results demonstrated that expression of hCRPs was sufficient to abrogate the need for systemic complement inhibition, but was not sufficient to prevent an induced antibody response and antibody-mediated rejection (AMR). When anti-Gal antibody was blocked *in vivo* using a Gal polymer, more consistent graft survival, median 35 days, was reported in pig-to-cynomolgus monkey hCXTx [30]. This study appears to be the first to detect an induced non-Gal antibody associated with xenograft rejection. These early CCS regimens were often poorly tolerated due to the narrow therapeutic index for cyclophosphamide.

An alternative immune suppression strategy based on induction with ATG and Rituximab and using tacrolimus and sirolimus maintenance immune suppression was used in WT;hCRP, GGTA-1 α-galactosyltransferase-deficient pigs (GTKO), and GTKO;hCRP transplants. The studies with WT;hCRP donors involved the largest series of transplants ($n = 63$) using a Gal polymer to test the effects of systemic anticoagulation and immune suppression on graft survival [31]. At moderate tacrolimus and sirolimus maintenance levels, hCXTx graft survival of up to 109 days (median survival 20 days) was achieved, similar to earlier results using CCS immune suppression. At higher levels of maintenance immune suppression [32, 33], maximal survival was improved (139 days) with more consistent and prolonged median survival of 96 days. This was the first instance of median cardiac xenograft survival in excess of 3 months. In these WT;hCRP studies, anti-Gal-mediated rejection was minimized and graft rejection was associated with non-Gal antibody. Moreover, testing three distinct, tightly controlled clinical anticoagulation therapies did not improve graft survival or affect the histology of graft rejection, indicating no strict requirement for, or benefit from, systemic anticoagulation. Using GTKO or GTKO;hCRP donors with moderate tacrolimus and sirolimus, immune suppression achieved comparable survival to earlier WT;hCRP transplants using Gal polymers, indicating that the adoption of GTKO donors obviated the need for specific therapy to control anti-Gal antibody and suggested that graft survival was limited by the effects of non-Gal antibody [34]. Also, the three-month median survival achieved in these hCXTx

studies made the conversion to oCXTx the appropriate model of choice moving forward.

3. Costimulation Blockade Immune Suppression

The more recent form of immune suppression utilized in hCXTx is costimulation blockade, primarily directed at the CD154 (CD40 ligand) and CD40 secondary signaling pathway, to block Th2 cell help for B cell activation. Antibody to CD154, originally shown to suppress allograft rejection [35], has been used extensively in pig-to-NHP hCXTx with donor organs ranging from WT;hCRP to GTKO;hCRP donors (Table 1). This immune suppression regimen was complex, including lymphocyte depletion with ATG and LoCD2b antibody, complement inhibition with CVF and steroids, and chronic postoperative immune suppression with mycophenolate mofetil and anti-CD154 antibody (5c8), and usually supplemented with a range of anticoagulant therapies. Early versions of this protocol also included pretransplant thymic irradiation [36] and used Gal polymers. With WT;hCRP donor hearts, maximal hCXTx organ survival was 139 days with a median of 23 days. More consistent organ survival was achieved (median 78 days) by transplantation of GTKO donor hearts into recipient baboons with little or no detected preformed non-Gal antibody [37]. In both instances, there was no apparent induction of circulating Gal or non-Gal antibody although the histology of the graft at explant, showing vascular antibody and complement deposition, was consistent with AMR. Importantly, anti-CD154 costimulation blocking regimens often reported complications with thrombocytopenia, consumptive coagulopathy (CC), and systemic inflammation which contributed to recipient loss [38, 39]. With GTKO;CD46 donor hearts, 236-day maximal graft survival (median survival 71 days) was achieved using a modified anti-CD154 protocol which included Rituximab induction to deplete B-cells [5]. Intensive postoperative monitoring in this study also likely contributed to prolonging graft survival. Explanted grafts showed evidence of ongoing humoral rejection; however, the authors indicated that survival was largely limited by nonimmune model-related issues for managing the recipient animals and recurrent thrombocytopenia, ascribed to the use of anti-CD154. The continued use of CVF, even with GTKO;CD46 donor organs, also contributes to systemic coagulation perturbations [40], precluding its clinical use.

Systemic thrombocytopenia and CC associated with anti-CD154 binding to activated platelets are well known, and significant efforts have been made in XTx models to find an effective substitute costimulation blockade regimen [41, 42]. Recent studies demonstrate that chronic administration of an anti-CD40 blocking antibody (2C10R4), substituting for anti-CD154, leads to prolonged hCXTx survival [25]. In the initial report, anti-CD40 (2C10R4), administered for just 60 days posttransplant, achieved maximal GTKO;hCRP hCXTx survival of 149 days and median survival of 84 days [25]. Graft survival appeared to be limited by the dosage and duration of immune suppression as withdrawal of anti-CD40 therapy resulted in a marked rise in antibody titer and xenograft rejection. Importantly, substituting anti-CD40 antibody for anti-CD154 moderated complications of thrombocytopenia and consumptive coagulopathy, which may also have contributed to improved graft survival. Using a higher dosage of anti-CD40 (2C10R4), administered for longer, resulted in longer survival of GTKO;hCRP donor hearts expressing human thrombomodulin (TBM) with maximal survival of 945 days (median survival 298 days) [8]. These outcomes likely underestimate survival as anti-CD40 therapy was reduced in 2 of 5 recipients after 100 days and was reduced for two other recipients after 1 year. In these latter recipients, anti-CD40 therapy was eventually withdrawn at 560 and 861 days posttransplant. In each case, reduction/cessation of anti-CD40 therapy resulted in induction of non-Gal IgM and IgG antibody with eventual graft rejection. Despite persistent vascular expression of human TBM, the histology of explanted rejected hearts exhibited features typical of xenograft rejection including thrombotic microangiopathy, vasculitis, intravascular thrombosis, and myocardial necrosis with little evidence of lymphocytic infiltration suggesting limited impact of the TBM addition. Recipients also received CVF, heparin, and aspirin.

The new anti-CD40-based costimulation blocking regimen appears to have achieved a level of humoral and cellular immune suppression which, for the duration that it is provided, blocks non-Gal AMR, with graft survival now measured in years. This appears to surmount a major obstacle to clinical XTx. While the current anti-CD40 (2C10R4) is a mouse/rhesus chimeric IgG4 antibody which would not be suitable for use in humans, it is reportedly being humanized [6]. There are also several other humanized anti-CD40 blocking antibodies under various levels of development (Table 2), suggesting there may soon be a clear path forward for immune suppression, using only approved therapies, to support clinical CXTx.

4. Donor Genetics

Genetic engineering of the donor pig is a cornerstone of XTx as it enhances organ survival and function, while reducing the need for systemic therapies in the recipient. The pace of genetic manipulation of the pig genome has significantly increased with the introduction of somatic cell nuclear transfer and sequence-directed nucleases [43, 44]. This proliferation currently outstrips the pace of analysis in pig-to-NHP transplants. There are now dozens of reported gene additions or deletions [45, 46] with suggestions that donor animals with 5 or more genetic alterations affecting complement regulation, antigen reduction, haemostatic incompatibilities, coagulation dysfunction, suppression of inflammation, adaptive T-cell immunity, and endogenous retrovirus infectious risks may be required for clinical XTx [47]. In the search for an "ideal" donor, the simultaneous introduction of multiple genetic modifications, without appropriately controlled experiments, may obfuscate their function, as well as introduce unnecessary complications. A consistent strategic approach to developing and testing new donor genetics would accelerate the application of clinical CXTx. Moreover, the accumulation of multiple gene modifications complicates

Table 2: Anti-CD40 antibodies in clinical development.

Antibody	Company	Status	Trial ID
SGN-40	Seattle Genetics Inc.	Phase 1 multiple myeloma	NCT00079716
		Phase 2 B-cell lymphoma	NCT00435916
		Phase 1/2 chronic lymphocytic leukemia	NCT00283101
ASKP 1240	Astellas	Phase 2 renal Tx phase 2 plaque	NCT01780844
		Psoriasis	NCT01585233
HCD122	Novartis	Phase 1, chronic lymphocytic leukemia	NCT00108108
		Phase 2 multiple myeloma	NCT00231166
		Phase 2 follicular lymphoma	NCT01275209
		Phase 1/2 Hodgkin's and non-Hodgkin's lymphoma	NCT00670592
Chi Lob 7/4	Cancer Res UK	Phase 1 cancer malignancies	NCT01561911
BG9588	NIDDK	Phase 2 renal Tx	NCT00001857
	NIAMS	Phase 2 lupus nephritis	NCT00001789

donor breeding programs to the point that somatic cell cloning may be required to maintain the genetic profile. This will increase cost and may limit the production of donor animals for preclinical studies, the results of which have already been frequently compromised by small group sizes.

Donor genetic modifications have focused on four main categories, antigen reduction [48], thromboregulation [49], immune suppression, and infectious disease [50]. Two additional non-Gal glycan antigens have been identified, N-glycolylneuraminic acid- (Neu5Gc-) modified oligosaccharides [51, 52] and the glycan product synthesized by porcine beta 1,4-N-acetylgalactosamine transferase-2 (B4GALNT2) [53]. Humans do not synthesize Neu5Gc due to a mutation in the CMP-N-acetylneuraminic acid hydroxylase gene (CMAH), but they do produce an array of antibodies which show Neu5Gc-dependent reactivity to sialylated oligosaccharides. These anti-Neu5Gc antibodies are noted for their role in serum sickness in patients treated with animal sera [54]. The B4GALNT2 gene catalyses the terminal addition of N-acetylgalactosamine (GalNAc) to a sialic acid-modified lactosamine acceptor producing GalNAcβ4[Neu5Acα2,3]-Gal β4GlcNAcβ3Gal, the SDa blood group antigen. This is an immunogenic glycan in pig-to-NHP CXTx [55]. Humans are known to produce low levels of IgM which bind the polyagglutinable human SDa blood group [56–59]. Targeted mutations affecting the porcine CMAH and B4GALNT2 genes have been made and combined with the GTKO mutation [48, 60–62]. These three mutations together minimized human IgM and IgG binding to porcine cells in over 90% of human serum samples [48] and reduced both IgM and IgG reactivity to background levels in 30% of allosensitized wait-listed renal transplant candidates [60]. Pig-to-NHP CXTx using organs with these mutations has not yet been reported. Minimizing tissue immunogenicity would appear to have obvious clinical benefit but demonstrating this in preclinical transplants will be difficult as NHPs do not produce anti-Neu5Gc antibody. Additionally, induction therapy with ATG, part of the current costimulation blocking immune suppression, may induce an anti-Neu5Gc antibody response in humans [63], which is not apparent in NHPs. This induced response could sensitize recipients and compromise Neu5Gc-positive donor organs in clinical CXTx. This suggests that new large animal transplant models, using CMAH-deficient recipients, may be required to test the pathogenicity of anti-Neu5Gc antibody and optimize the use of biological agents for immune suppression. Despite these issues, genetic engineering directed at reducing the antigenicity of porcine tissue is likely to significantly impact clinical CXTx.

Interest in transgenic augmentation of thromboregulation stems primarily from recognition of molecular incompatibilities between porcine and human TBM [64]. Secondarily, immune-independent recipient and donor cell-to-cell interactions have been described *in vitro* which are proposed to contribute to donor endothelial cell activation [65, 66], systemic haemostatic dysfunction, and CC [67–69]. Several groups have reported production of human TBM transgenic pigs [70–72], and *in vitro* analysis of porcine endothelial cells expressing human TBM shows that it alleviates the molecular incompatibility with efficient production of activated human protein C [64, 73]. A limited number of pig-to-NHP CXTx studies with human TBM expressing donor organs have been reported [8, 74]. The impact of human TBM expression cannot be determined from these studies as their designs lacked controls without TBM, included chronic systemic heparin administration, and had no direct measure of human TBM function. Transgenic expression of other key components which affect haemostasis, CD39, CD47, TFPI, and EPCR, has also been reported [12, 75].

The common donor modifications, transgenic expression of hCRPs, and the GTKO mutation have been thoroughly analysed and validated. Physiological or supraphysiological vascular expression of one or more human complement regulatory genes, CD59, CD55, or CD46, establishes an intrinsic barrier which regulates the complement cascade [76–78], reduces the incidence of hyperacute rejection, and limits complement-mediated injury [15]. Likewise, the significance of anti-Gal antibody and benefit of targeted mutation of the porcine α-galactosyltransferase locus [79–81] have been extensively documented in *in vitro* [82] and *in vivo* studies in both pig-to-NHP [83–86] and GTKO mouse models [87–91]. The combination of hCRP and GTKO donor modifications has also been specifically examined and demonstrated to be beneficial, preventing rare hyperacute

GTKO hCXTx rejection and early immune injury [34, 92]. This basic genetic background, GTKO;hCRP, represents, in our judgement, the current, proven starting base for any clinical study. Additional CMAH- and B4GALNT2-directed antigen reduction would appear to be a beneficial clinical priority.

5. Orthotopic CXTx and Perioperative Graft Function

The vast bulk of CXTx studies have to date utilized an abdominal non-life-supporting heterotopic transplant model where the graft is contractile but does not support the recipient's circulation. In comparison, there have been a limited number of oCXTx studies [9–11, 93–99]. These studies, predominantly using WT;hCRP donors, report healthy recipient survival up to 57 days [46]. In this difficult model, recipient death is often due to postoperative management limitations, with explanted hearts often showing little histologic evidence of significant rejection. These studies, which could not yet utilize the most recent highly successful costimulation blockade immune suppression, clearly indicate that porcine hearts can provide life-sustaining and adequate circulation to NHPs and suggest that the efficacy of oCXTx is not intrinsically limited by cardiac function but by immune rejection and postoperative management. To demonstrate life-supporting oCXTx in a preclinical NHP model, consistent with the ISHLT guidelines, will require not only effective immune suppression and appropriate donor genetics but also substantial clinical level resources, expertise, and postoperative management.

Orthotopic CXTx studies also unmasked a potential impediment to clinical CXTx which was not apparent in hCXTx studies. Every research group that has performed oCXTx has reported variable perioperative mortality ranging from 40 to 60% within the first 48 hours. Xenograft failure in this time period was not due to hyperacute rejection as the explanted hearts show vascular antibody deposition but otherwise normal myocardial histology [100]. Instead, early graft failure was associated with primary organ dysfunction. We have called this phenomenon perioperative cardiac xenograft dysfunction (PCXD) which at this high frequency currently represents a significant barrier to clinical CXTx. Our ongoing studies suggest that PCXD is similar to ischemia reperfusion injury or cardiac stunning. We find that PCXD can be modulated with a preconditioning regimen to reduce circulating antibody, B-cells, and plasma cells prior to transplant, coupled with improved organ preservation [101]. In recipients which survive beyond 48 hours, PCXD is less evident and echocardiographic analysis indicates that PCXD is completely reversible showing that the normal cardiac reparative processes function across the XTx barrier [11]. Intrathoracic heterotopic cardiac transplantation, where both the donor and recipient hearts contribute to the circulation, was successfully used in early allotransplantation when techniques for donor organ preservation were being optimized. Preclinical intrathoracic heterotopic cardiac xenotransplantation studies, although complex, potentially offer a unique opportunity to study the aetiology and recovery from PCXD [102, 103]. Genetic engineering approaches may also have the potential to mitigate PCXD, for example, by providing high levels of CD39 expression [75] or reducing sodium hydrogen ion exchange activity [104].

6. Diagnosis and Treatment of Rejection

The ability to diagnose and treat rejection is a key component of clinical transplantation. The ISHLT has developed pathologic grades (pAMR1–3) of immunopathologic features of endomyocardial biopsies which along with graft dysfunction and levels of donor-specific antibody are used for the diagnosis AMR in cardiac allotransplantation [105]. The most severe pathology (pAMR3), associated with significant graft dysfunction and poor clinical outcomes, can be treated with a combination of increased and optimized immune suppression, depletion of circulating antibody, and IVIg. More aggressive salvage therapies may also include B-cell and plasma cell depletion and complement inhibition [105, 106]. In CXTx, there are few studies which have attempted to diagnose and treat presumptive rejection episodes, most of these after hCXTx [9, 25, 33, 107–109]. Putative rejection episodes were diagnosed based on biochemical markers (troponin T, AST), graft contractility, telemetric measures of cardiac performance, and echocardiography. Serial biopsies after oCXTx will likely be applicable for diagnosis of rejection [109–111], but the difficulty of obtaining endomyocardial biopsies in NHPs has limited their exploration in animal models. When presumptive rejection episodes were treated using steroids, or steroids and ATG, there was no evidence for reversal of rejection, and, unsurprisingly, in some instances, excessive antirejection therapy increased the frequency of infectious complications [9, 25]. Effective therapies to reverse AMR in XTx remain to be fully explored.

Based on the high frequency of AMR, the wide diversity of potential polymorphic porcine peptides and the chronic detection of vascular antibody deposition in GTKO donor hearts, it is necessary to establish methods for early diagnosis and effective treatment. It appears that anti-CD40-based immune suppression, which is likely to be used in clinical CXTx, relies heavily on effective costimulation blockade, as withdrawal of anti-CD40 therapy has resulted in the induction of non-Gal IgM and IgG [8]. This has at least two potential consequences. Firstly, costimulation blockade complicates the use of plasmapheresis, commonly used to treat AMR, as it would remove both therapeutic and pathogenic antibodies. Secondly, chronic dosing with biological therapeutics risks the development of anti-antibody immune responses [112, 113]. While the frequency of this response is difficult to estimate and cannot be safely extrapolated from the results reported for other antibody-based therapeutics, in the context of clinical CXTx, an anti-anti-CD40 response would be potentially serious. Few clinical methods can reverse antibody-mediated heart rejection so it will be important, prior to clinical CXTx, to develop and test, to as great a degree as possible, xenospecific therapies for detecting and treating AMR using a pig-to-NHP transplant model. This may include alternative versions of anti-CD40 (Table 2), alternative costimulation strategies [13], total lymphoid

radiation [114], or current antibody reduction therapies [105, 106]. Ideally, such a study would be performed using CXTx, but life-supporting kidney XTx, with well-known physiological markers for organ function, maybe a more pragmatic solution as ongoing kidney rejection will not result in rapid recipient death. What is clear to investigators with experience in oCXTx is that the NHP model plays a critical role in progressing to clinical application, but has intrinsic limitations with particular regard to recipient management.

7. Infectious Disease Issues

Complete knowledge and risk-free application of clinical CXTx, as with most major advances in medicine, retains elements of uncertainty. The potential for disease transmission has been a significant concern for clinical XTx [115]. Concern has been expressed about the potential of porcine endogenous retroviruses (PERV) to emerge in XTx recipients, infect patient tissues, and adapt to humans [116]. Since this potential was identified, molecular and immunologic assays to monitor PERV infection have been developed [117], and significant advances have been made in mapping PERV proviral sites [118, 119] and understanding the basic biology of PERV infection [120]. Several clinical studies of patients exposed to porcine tissues [121–124] or in NHP XTx recipients [125] have also failed to detect PERV infection. The generation of high titer human-trophic PERV requires the recombination of relatively rare PERV-C proviral sequences with more common PERV-A. Selective breeding can be used to eliminate PERV-C from donor pigs [126]. Alternatively, nuclease-directed mutation of the PERV *pol* gene has been shown to induce widespread PERV proviral deletions [50], but this may be unnecessary if PERV-C is eliminated by selective breeding. While diligent monitoring of PERV infection in XTx recipients is prudent, the apparent risk presented by PERV appears to be small and is unlikely to delay clinical CXTx. Aside from endogenous retrovirus, specific pathogen-free (SPF) donor pig facilities have been produced and populated with caesarean-derived piglets. Some of these sites have been operational and breeding pigs for many years demonstrating the feasibility to routinely produce donor pigs with exceptionally high health standards.

8. Conclusion

It is clear that cardiac and renal XTx can benefit patients in need of organ replacement. If continued studies as outlined above are performed, we are optimistic that this technology will soon be ready for clinical testing. These remaining key preclinical studies are required to ensure the efficacy and safety of clinical CXTx. Principally a life-supporting preclinical oCXTx study in NHPs must be performed to demonstrate acceptable perioperative and postoperative recipient survival. This study should optimize organ preservation and utilize immune suppression based on anti-CD40 costimulation blockade. To meet ISHLT suggested standards [14], this study, involving at least 16 CXTx recipients, will require significant financial resources, an infrastructure to simultaneously maintain multiple CXTx postoperative recipients and a dedicated team of clinicians, veterinarians, scientists, and animal technologists. Donor organs should minimally contain GTKO;hCRP genetics, likely with additional antigen reduction of CMAH-KO and B4GALNT2-KO. Clinical use of additional genetic modifications should be founded on further rigorous preclinical testing in NHPs to demonstrate their utility, which, in the case of CMAH, will likely require testing in an immunologically appropriate CMAH-KO large animal transplant model. As well as achieving adequate perioperative and postoperative recipient survival, such a preclinical study should allow for organ rejection. This rejection study, while unlikely to fully predict the clinical immune response, will suggest essential xenospecific assays and therapies to reverse rejection which can be further refined during clinical CXTx.

Genetic engineering has significantly improved CXTx organ survival but ongoing creation of new genetics, in pursuit of the perfect donor, has the potential to delay clinical studies. We believe the initial clinical studies will rely primarily on known systemic immune suppression and genetics and that further optimization of donor genetics is best pursued in response to identified, researched clinical immune and physiological requirements.

Dependent on the results of these prospective preclinical oCXTx studies in NHPs, it appears to us that the era of clinical CXTx is approaching. The scientific path forward is demanding but well defined; however, the complexity of any clinical XTx program, including the heart, presents a substantial and unique set of regulatory challenges which need to be addressed expeditiously to avoid delaying the realization of clinical use.

Abbreviations

AMR: Antibody-mediated rejection
B4GALNT2: Beta 1,4-N-acetylgalactosamine transferase-2
CC: Consumptive coagulopathy
CCS: Cyclophosphamide, cyclosporine, and steroids
CMAH: CMP-N-acetylneuraminic acid hydroxylase
CVF: Cobra venom factor
CXTx: Cardiac xenotransplantation
GTKO: Alpha-galactosyltransferase (GGTA_1) mutant
hCRPs: Human complement regulatory proteins
hCXTx: Heterotopic CXTx
ISHLT: International Society of Heart and Lung Transplantation
Neu5Gc: N-Glycolylneuraminic acid
NHP: Nonhuman primate
oCXTx: Orthotopic CXTx
PCXD: Perioperative cardiac xenograft dysfunction
PERV: Porcine endogenous retroviruses
RXTx: Renal xenotransplantation
SPF: Specific pathogen free
TBM: Thrombomodulin
WT: Gal-positive pig
XTx: Xenotransplantation.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Review Article

The Role of Costimulation Blockade in Solid Organ and Islet Xenotransplantation

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Pig-to-human xenotransplantation offers a potential bridge to the growing disparity between patients with end-stage organ failure and graft availability. Early studies attempting to overcome cross-species barriers demonstrated robust humoral immune responses to discordant xenoantigens. Recent advances have led to highly efficient and targeted genomic editing, drastically altering the playing field towards rapid production of less immunogenic porcine tissues and even the discussion of human xenotransplantation trials. However, as these humoral immune barriers to cross-species transplantation are overcome with advanced transgenics, cellular immunity to these novel xenografts remains an outstanding issue. Therefore, understanding and optimizing immunomodulation will be paramount for successful clinical xenotransplantation. Costimulation blockade agents have been introduced in xenotransplantation research in 2000 with anti-CD154mAb. Most recently, prolonged survival has been achieved in solid organ (kidney xenograft survival > 400 days with anti-CD154mAb, heart xenograft survival > 900 days, and liver xenograft survival 29 days with anti-CD40mAb) and islet xenotransplantation (>600 days with anti-CD154mAb) with the use of these potent experimental agents. As the development of novel genetic modifications and costimulation blocking agents converges, we review their impact thus far on preclinical xenotransplantation and the potential for future application.

1. Introduction

Organ transplantation remains the definitive treatment for patients suffering from end-stage organ failure. Unfortunately, this treatment remains severely limited due to the critical shortage of suitable allografts for transplantation [1, 2]. The use of genetically engineered pigs as a supplemental source of tissues or organs offers a promising answer to this dilemma [3]. Pig-to-human xenotransplantation has been pursued for more than a century; however, early studies demonstrated substantial barriers to clinical application in the form of hyperacute rejection, acute humoral xenograft rejection (AHXR), and thrombosis [4, 5].

The modern era of xenotransplantation was stimulated by the identification of the Gal α(1,3) Gal (Gal) porcine epitope and its role in early rejection [6–8]. The subsequent advent of α1,3-galactosyltransferase gene knockout (GTKO) pigs eliminated a major barrier to xenotransplantation by negating the role of high percentage of human xenoreactive antibodies [9, 10]. However, residual preformed human antibodies to GTKO pig antigens suggested additional major barriers (i.e., anti-non-Gal antibodies), which would hinder progress towards clinical application. Nevertheless, this remains a major breakthrough as the identification of Gal and production of GTKO pigs demonstrated the potential of reducing porcine antigenicity through genetic modification.

The initial production of GTKO animals was performed through a tedious process of homologous recombination; however, recent advances in gene editing have dramatically sped the pace of xenotransplantation research (Table 1) [9, 11–13] setting the stage for highly efficient and rapid porcine genetic modification. Recently, the role of genetically engineered pigs has been reviewed, and this role effectively negates the human anti-pig humoral response to the threshold where hyperacute rejection and AHXR are no longer expected [9, 12–14]. In this climate of reduced *humoral*

Table 1: Timeline for application of evolving techniques for genetic engineering of pigs employed in xenotransplantation.

Year	Technique
1992	Microinjection of randomly integrating transgenes
2000	Somatic cell nuclear transfer (SCNT)
2002	Homologous recombination
2011	Zinc finger nucleases (ZFNs)
2013	Transcription activator-like effector nucleases (TALENs)
2014	CRISPR/Cas9

CRISPR/Cas9: clustered randomly interspaced short palindromic repeats and the associated protein 9 (table adopted from Cooper et al.) [9].

xenoantigenicity, an appraisal of pharmacologic strategies that will modulate the human *cell-mediated* response to porcine xenografts is increasingly relevant.

The cell-mediated response in allotransplantation is addressed with an effective pharmacologic armamentarium, mainly with calcineurin or mTOR inhibitors [15, 16]. Today, one of the most active frontiers in immunology and transplantation research is T cell costimulation signal modification. Much work over the past decade has defined costimulation signals, which regulate T cell activation and immune tolerance [17]. Although most of these agents are still experimental and early in the development pathway, preclinical studies utilizing experimental costimulation blockade agents have demonstrated prolonged engraftment of both solid organ and islet xenografts [18–25]. The approval of LEA29Y (belatacept) as a CTLA4-Ig protein for use in renal allotransplantation brought costimulation blockade to the clinic in the early 2000s [26, 27]. This was made possible after promising results from belatacept administration in preclinical nonhuman primate studies [28, 29].

In the last decade, researchers have increasingly utilized pig-to-nonhuman primate xenotransplantation models to study novel xenograft modification and novel costimulatory immunosuppression strategies in parallel. As discussions of pig-to-human xenotransplantation trials are underway [30, 31], we herein provide an overview of costimulation pathways, the current standing of clinical and preclinical development of these agents, and the preclinical data regarding their use in xenotransplantation.

2. T Cell Regulation through Costimulation Pathways

The adaptive immune system generates targeted responses first through (i) T cells identifying the antigen of interest and (ii) supplementary stimuli in the form of costimulation to induce antigen-specific T cell proliferation. Without these adjunct signals, T cells become anergic or undergo apoptosis and thus the response against that antigen is abrogated [32]. In this way, costimulation pathways support the role of T cell receptors (TCRs)—major histocompatibility complex (MHC) interaction by providing T cell the context of the antigen. Secondary and tertiary signals driven by cell surface costimulation molecules and soluble cytokines, respectively, determine the parameters of T cell activation [33]. Cytokines produced by the antigen-presenting cell (APC) and the T cell itself further propagate this activation cascade to induce a robust T cell response. Conventional immunosuppression works to abrogate the TCR and cytokine-induced signaling pathways preventing T cell activation [15, 16]. However, their lack of specificity to T cell mechanisms has led to well-recognized adverse side effects.

Costimulation pathways for T cell activation occur through a unique subset of cell surface markers, which are highly specific for the immune system and thus provide a target for immune modulators. Figure 1 depicts the most commonly studied costimulation signals for potential use in transplant applications. The interaction of CD28 with CD80/CD86 has been the best defined. CD28 is highly expressed on naïve T cells. During TCR engagement with an APC, binding of CD28 to CD80/CD86 results activation and proliferation of the T cell. A feedback mechanism occurs at this juncture by which CD28 is then downregulated and the T cell increases expression of CTLA4-Ig. This molecule binds CD80/CD86 with much higher affinity than CD28 and produces an inhibitory signal as a highly evolved feedback mechanism [34].

Another increasingly significant costimulation pathway is the CD40/CD154 (CD40 ligand) interaction, which has been shown to be a potent stimulator of T and B cell activation through conventional APC interactions and also through interactions with innate immune cells and endothelium [35–38]. The inducible T cell costimulator (ICOS) molecule (CD278) has more recently been discovered to play an important role in T cell activation and differentiation as well as T and B cell interactions [39].

These costimulation pathways play a significant role during antigen recognition and T cell activation. Activated T cells rely on a specialized repertoire of surface proteins that assist in migration, adhesion, and interactions across the immunologic synapse to facilitate their effector function [40]. Lymphocyte function-associated antigen 1 (LFA1) is a well-studied molecule known to assist in immune cell endothelial attachment and migration and is recognized to play an important role in the stabilization of the immunologic synapse during antigen recognition and effector function (Figure 1) [41–43]. CD2 is more constitutively expressed on memory T cells, and interaction with LFA-3 is thought to not only have migration functions but also act as an activator of the potent memory T cell proliferation and response [40].

3. T Cell Costimulation in Organ Allotransplantation

Costimulation blockade has been extensively studied in preclinical allotransplantation models [41, 44–50]. Their relevance to xenotransplantation and xenoimmunity requires a thorough understanding of the salient findings from this growing body of research. One of the initial costimulation blockade agents was CTLA4-Ig, a protein that binds CD80/CD86 thus preventing CD28 costimulation and T cell activation. Preclinical data for CD40/CD154 blockade using anti-CD154 mAb also emerged in parallel with promising

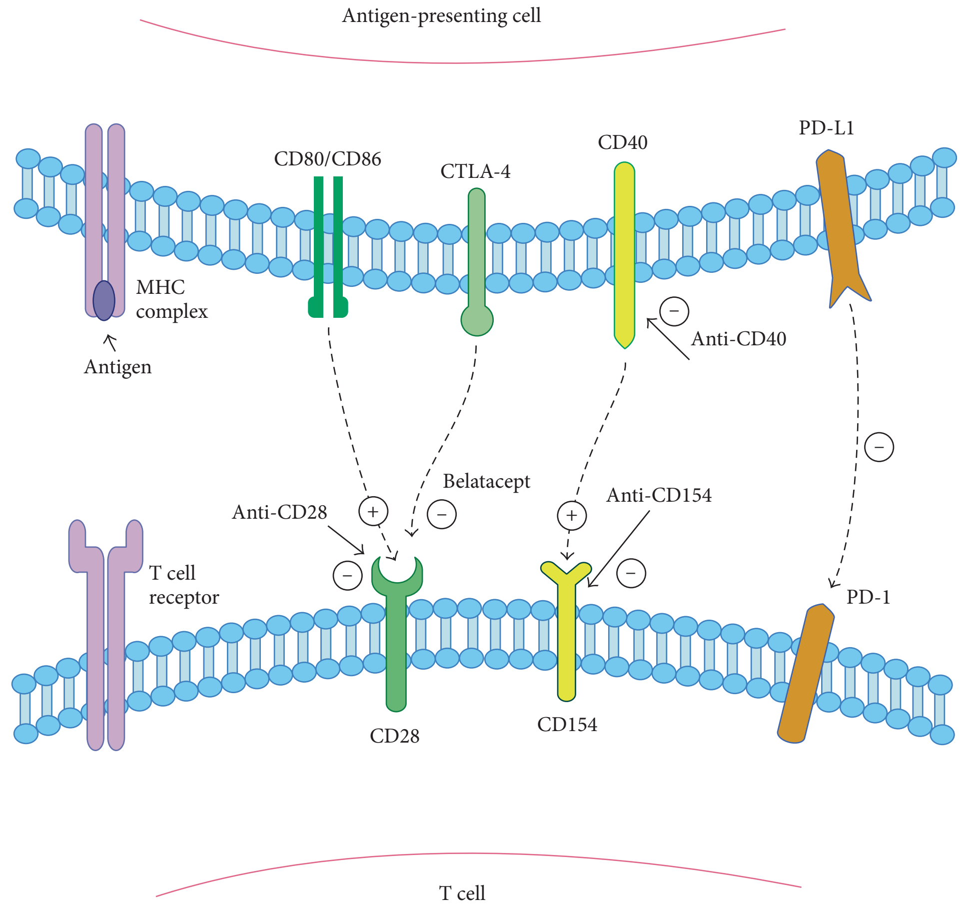

Figure 1: Costimulation pathways in T cell regulation. Upon MHC-antigen interaction with the TCR, costimulation pathways can augment or suppress the activation of the T cell. From left to right, CD28 is activated by CD80/CD86; however, after T cell activation, CTLA-4 is upregulated and with higher affinity than CD80/CD86 and binds to CD28 inhibiting the signal. CTLA-4Ig and belatacept work by taking advantage of their higher affinity to CD28 over CD80/CD86 and thereby block CD80/CD86 activation of CD28. CD154 and CD40 are other potent activators of T cells; monoclonal antibodies against either of these surface proteins have potential for application in transplant immunosuppression. PD-1 is expressed on T cells, and interaction with PD-1 Ligand (PD-L1) produces a suppressive signal to the T cell.

results. For example, an earlier study utilizing CTLA4-Ig and an anti-CD154 mAb (5C8 molecule) demonstrated synergistic prolongation of allograft survival in a nonhuman primate model, which continued even after withdrawal of immunosuppression [44]. Blockade of CD40/CD154 signaling pathway also was able to prolong graft survival in both renal and islet allotransplantation in nonhuman primates [44, 46, 51]. In these studies, the combination of both CTLA4-Ig and CD40 blockade appeared to prevent donor-specific antibody formation.

Memory T cells have been implicated in belatacept-resistant rejection; therefore, adjuvant therapy targeting memory T cell-specific features has been studied [40, 52]. An initial study of the LFA-3Ig molecule (alefacept) *in vitro* demonstrated suppression of alloreactive memory T cells, which were not suppressed by belatacept alone [45, 53]. Studies in nonhuman primates, however, demonstrated minimal benefit with an increased incidence of infectious complications [45, 47, 48, 53]. Based on early data, clinical use of the LFA-1 inhibitor, efalizumab, demonstrated some benefit in islet transplantation based on early data [42]. The use of LFA-1 inhibitor in combination with costimulation blockade also appeared to further prolong graft survival in islet allotransplantation [54]. LFA-1 exists in two forms: a commonly expressed, low-affinity form and a transient, high-affinity form, expressed only during activation. A recent study examined the use of more specific LFA-1 inhibitors (leukotoxin A and AL-579); targeting the high-affinity form of LFA-1 also did not demonstrate additional benefit in a renal transplant model [43]. Despite these data and the clinical potential, both alefacept and efalizumab were removed from the market by their manufacturers precluding further clinical study. A study using ICOS blockade with belatacept did not demonstrate any visible benefit to the combination of the two [50].

Costimulation blockade in clinical transplantation was first successfully introduced with the use of belatacept, a CTLA4-Ig molecule with higher affinity for B7 [26]. The initial BENEFIT trials demonstrated similar efficacy of

belatacept-based regimens versus calcineurin inhibitors with an improved side effect profile [55–58]. However, a higher number of patients experienced an early severe rejection, which led to hesitation by many clinicians for widespread use [59]. Most of these rejection episodes were medically reversible which led to similar graft survival rates. The sparing of renal function demonstrated a potential benefit in long-term graft survival. Interestingly, patients who were on belatacept therapy also lacked significant production of donor-specific antibodies [29]. Further investigation into belatacept-resistant rejection demonstrated specific subsets of memory T cells that were present in patients who were not responsive to belatacept [40, 52, 60–62]. Alternative regimens incorporating belatacept in addition to conventional agents have shown promise [63–65], and further study to risk stratify these patients to individualize and introduce adjuvant therapy is ongoing.

Phase I clinical trials of a CD154 inhibitor demonstrated increased thrombotic phenomena not identified in preclinical testing and thus prevented clinical approval [66, 67] (as was subsequently demonstrated in xenotransplantation [68]). As preclinical data in allotransplant models appeared promising, newer agents to inhibit the CD40/CD154 and CD28/CD80/CD86 interaction and other costimulatory pathways are in the pipeline [69–72] but will need to complete their drug development cycle prior to consideration for human xenotransplant trials.

4. Costimulation Blockade in Xenotransplantation

The past two decades have been marked by great advances in the field of xenotransplantation with unprecedented graft survival times seen in preclinical models [1, 5, 13]. Tables 2, 3, and 4 summarize selected studies in solid organ (heart, kidney, and liver) and islet xenotransplantation with a specific use of anti-CD154mAb (Table 2), anti-CD40mAb (Table 3), or CTLA4-Ig (Table 4) between 2000 (the first use of costimulation blockade in xenotransplantation) to 2017. Continued development and improvement upon immunosuppressive regimens and the introduction of novel experimental agents appear to have contributed to this progress. Studies from the early part of the previous decade showed that induction therapy followed by high-dose conventional combination maintenance regimens was generally (but not uniformly) sufficient to sustain life-supporting pig grafts in nonhuman primates [73]. Conventional immunosuppressive therapy included agents such as cyclophosphamide, cyclosporine, mycophenolate mofetil, methylprednisone, and prednisolone (Tables 2, 3, and 4).

In 2000, Buhler et al. introduced the concept of costimulation blockade to the field of xenotransplantation [74]. Using a murine anti-human CD154mAb, they attempted to induce immune tolerance in nonhuman primates to transplanted pig peripheral blood mononuclear cells (PBMCs). More preclinical studies followed in both solid organ and islet xenotransplantation (Table 2) and increased markedly in the following decades. The most studied costimulatory modifiers within xenotransplantation have included anti-CD154mAb (Table 2), anti-CD40mAb (Table 3), and the CD28/B7 pathway (including CTLA4-Ig proteins abatacept and belatacept, as well as anti-CD28mAb, Table 4). Anti-CD154mAb therapy significantly prolongated porcine renal xenograft survival in nonhuman primates, with recent data demonstrating survival up to 405 days [22, 75, 76]. Unfortunately, this therapy is unlikely to be available for clinical xenotransplantation trials in the near future due to the agent's known thrombogenic properties [66–68]. High avidity CTLA4-Ig (belatacept) through interrupting the CD28/B7 pathway may be insufficient as monotherapy for xenograft maintenance [77]. Anti CD40mAb-based regimens have contributed to some of the longest reported xenograft survivals of pig heart and livers [24, 78]. Adhesion blockade with LFA-1 has also been utilized in a model of xenogenic islet transplantation, but with minimal benefit [79]. Further study continues in preclinical models to identify the most effective combination of costimulation blockade for xenotransplantation.

5. Costimulation Blockade and Genetic Modification of the Pig

Moving in parallel with this growing interest in xenotransplant costimulatory modification, *genome*-editing strategies aimed at costimulation pathways has also gained momentum. Xenotransplantation offers the unique potential to incorporate modifiers of the host immune response within the graft expression profile itself. To date, genetically modified pigs have been produced that alter the expression of endogenous porcine CTLA-4-Ig [80], or LEA29Y [81], or express human CD39 [82], or a human dominant-negative mutant class II transactivator [83]. Exhibiting variable successes, these approaches incorporate inhibitory regulation of the host costimulation interactions within the graft itself with the goal of facilitating suppression of host immune tolerance to the xenograft with less pharmacologic intervention than is required for allografts.

Regarding islet xenotransplantation, to date, five independent groups have reported survival of pig islets (genetically engineered or wild-type) for more than 3 months after transplantation into the liver of a nonhuman primate [19, 84]. Four groups utilized anti-CD154mAb-based immunosuppressive therapy (Table 2). Due to the likely unavailability of this agent, the Emory group has tried novel strategies with other clinically applicable or potentially clinically applicable medications such as basiliximab (anti-CD25mAb), LFA-1 blockade, and anti-CD40mAb (Table 3), in combination with belatacept.

Although several of these genetic strategies have provided promising results, the majority of gene-modification models are aimed at xenoantigen removal, complement regulation, or thromboregulatory properties of the xenograft. Indeed, these advances in genome-editing techniques have catalyzed a recent influx of novel and unique genetic backgrounds to the field of xenotransplantation. This rapid development raises a significant experimental issue; both novel genomic strategies and experimental immunosuppression strategies warrant individual appraisals. In the absence of a unified

Table 2: Selected studies using anti-CD154mAb in pig-to-nonhuman primate xenotransplantation.

First author (year)	Donor pig	Recipient NHP	Immunosuppressive regimen	Longest survival (days)
		Heart xenotransplantation, heterotopic		
Buhler (2000) [86]	WT	Baboon	TBI, TI, splenectomy, IA, ATG, CVF, CSA, or anti-CD154mAb, MMF +/− pig stem cells	N.A
Houser (2004) [87]	CD55	Baboon	ATG, anti-CD2mAb, TI, CVF, anti-CD154mAb, MMF, CS	139
Dor (2005) [88]	GTKO	Baboon	ATG, anti-CD154mAb, MMF, CS	179
Kuwaki (2005) [89]	GTKO	Baboon	ATG, anti-CD2mAb, TI, CVF, anti-CD154mAb	179
Wu (2005) [90]	CD46	Baboon	ATG, anti-CD154mAb, +/− anti-CD20mAb +/− CTLA4-Fc	11
Wu (2007) [91]	CD46	Baboon	ATG, anti-CD154mAb, GAS194 or TPC, +/− IA	36
Ezzelarab (2009) [92]	GTKO	Baboon	ATG, CVF, anti-CD154mAb, MMF, CS	56
Mohiuddin (2012) [93]	GTKO.CD46	Baboon	ATG, anti-CD20mAb, anti-CD154mAb, MMF, CS	236
Kim (2013) [94]	GTKO	Cynomolgus	ATG, anti-CD20mAb, anti-CD154mAb, tacrolimus, CS	24
Ezzelarab (2015) [95]	GTKO	Baboon	ATG, anti-CD154mAb, MMF	56
Iwase (2015) [96]	GTKO.CD46.TBM	Baboon	ATG, anti-CD20mAb, anti-CD154mAb, MMF, CS	52
		Kidney xenotransplantation		
Buhler (2000) [86]	WT	Baboon	TBI, TI, splenectomy, IA, ATG, CVF, CSA, or anti-CD154mAb, MMF +/− pig stem cells	N.A
Buhler (2001) [97]	CD55	Baboon	TBI, TI, splenectomy, IA, ATG, CVF, anti-CD154mAb, MMF, CS	29
Barth (2003) [98]	CD55	Baboon	Thymokidneys, anti-CD2mAb, ATG, anti-CD154mAb, CyP, CVF, MMF, CS	229
Gollackner (2003) [99]	CD55	Baboon	TI, splenectomy, IA, ATG, anti-CD154mAb, CyP, CVF, MMF, CS	13
Knosalla (2003) [100]	CD55	Baboon	TI, splenectomy, IA, ATG, anti-CD154mAb, CyP, CVF, MMF, CS	29
Yamada (2005) [75]	GTKO	Baboon	Vascularized thymic lobe, WBI, anti-CD2mAb, anti-CD154mAb, MMF, CS, CVF	68
Shimizu (2005) [101]	CD55	Baboon	Thymokidneys, splenectomy, IA, anti-CD3mAb, ATG, anti-CD154mAb, CyP, CVF, MMF	30
Griesemer (2009) [102]	GTKO	Baboon	Thymectomy, splenectomy, TBI, ATG, anti-CD2mAb, anti-CD154mAb, tacrolimus, MMF, anti-CD20mAb	83
Lin (2010) [103]	GTKO.CD46	Baboon	ATG, antiCD154mAb, MMF, CVF, CS	16
Nishimura (2011) [104]	GTKO	Baboon	Thymokidney, thymectomy, splenectomy, anti-CD3, antiCD2mAb, ATG, anti-CD20mAb, tacrolimus, MMF, anti-CD154mAb	15
Ezzelarab (2015) [95]	GTKO	Baboon	ATG, anti-CD154mAb, MMF	10
Higginbotham (2015) [22]	GTKO.CD55	Rhesus	Anti-CD4, anti-CD8, anti-CD154mAb, MMF, CS	310
Kim (2017) [76]	GTKO.CD55	Rhesus	Anti-CD4, anti-CD8, anti-CD154mAb, MMF, CS	405
		Liver xenotransplantation		
Kim (2002) [105]	GTKO	Baboon	ATG, LoCD2b, CVF, anti-CD154mAb, azathioprine, tacrolimus, CS	9
Navarro-Alvarez (2016) [106]	GTKO	Baboon	ATG, LoCD2b, CVF, anti-CD154mAb, tacrolimus, CS	6

Table 2: Continued.

First author (year)	Donor pig	Recipient NHP	Immunosuppressive regimen	Longest survival (days)
		Islet xenotransplantation		
Buhler (2002) [18]	WT	Baboon	Splenectomy, IA, TBI, ATG, CVF, anti-CD154mAb, CSA, MMF, CS	28
Hering (2006) [107]	WT	Cynomolgus	Anti-CD25mAb, FTY720, rapamycin, anti-CD154mAb	187
Cardona (2006) [108]	WT	Rhesus	Anti-CD25mAb, anti-CD154mAb, CTLA4-Ig	>260
Rood (2007) [109]	GTKO	Cynomolgus	ATG, CVF, anti-CD154mAb, MMF, tacrolimus	>58
Casu (2008) [110]	WT	Cynomolgus	ATG, anti-CD154mAb, MMF	>60
van der Windt (2009) [19]	CD46	Cynomolgus	ATG, anti-CD154mAb, MMF	396
Thompson (2011) [20]	GTKO	Rhesus	Anti-CD154mAb, anti-LFA1mAb, MMF, belatacept	249
Bottino (2014) [111]	GTKO.CD46. TFPI.CTLA4Ig.CD39	Cynomolgus	ATG, MMF, anti-CD154mAb, CS	365
Shin (2015) [112]	WT	Rhesus	Anti-CD154mAb, ATG, rapamycin, CVF, adalimumab	>603

ATG: antithymocyte globulin; CS: corticosteroids; CSA: cyclosporine A; CVF: cobra venom factor; CyP: cyclophosphamide; NHP: nonhuman primate; TBI: total body irradiation; TI: thymus irradiation; mAb: monoclonal antibody; MMF: mycophenolate mofetil; mAb: monoclonal antibody; GTKO: α1,3-galactosyltransferase gene knockout; GAS914: a soluble glycoconjugate comprising Gal on poly-L-lysine backbone; N.A: not applicable; TBM: thrombomodulin; TPC: an aGal-polyethylene glycol polymer conjugate; WT: wild-type.

Table 3: Selected studies using anti-CD40mAb in pig-to-nonhuman primate xenotransplantation.

First author (year)	Donor pig	Recipient NHP	Immunosuppressive regimen	Longest survival (days)
		Heart xenotransplantation, heterotopic		
Iwase (2015) [96]	GTKO.CD46.TBM	Baboon	ATG, belatacept, anti-CD40mAb, tacrolimus, MMF, CS	130
Mohiuddin (2016) [78]	GTKO.CD46.TBM	Baboon	ATG, anti-CD20mAb, anti-CD40mAb, CS	>900
		Kidney xenotransplantation		
Iwase (2015) [23]	GTKO.CD46.CD55 TBM.EPCR.CD39	Baboon	ATG, anti-CD20mAb, anti-CD40mAb, rapamycin, tocilizumab, etanercept	136
		Liver xenotransplantation		
Shah (2017) [24]	GTKO	Baboon	ATG, anti-CD40mAb, tacrolimus, CVF, CS	29
		Islet xenotransplantation		
Thompson (2011) [21]	WT	Rhesus	Anti-CD25mAb, anti-CD40mAb, rapamycin, belatacept	203

NHP: nonhuman primate; WT: wild-type; ATG: antithymocyte globulin; CVF: cobra venom factor; MMF: mycophenolate mofetil; mAb: monoclonal antibody; CS: corticosteroids; GTKO: α1,3-galactosyltransferase gene knockout; TBM: thrombomodulin; EPCR: endothelial cell protein C receptor.

approach to gene modification within xenotransplantation, a cohesive appraisal of costimulatory intervention is challenging. The heterogeneity of genetic background thus prevents an effective stratification of costimulation blockade strategies for xenotransplantation. At present, a combination of graft modifications and exogenous immunosuppressive therapy to the host will be necessary to promote clinical application of xenotransplantation [1, 3, 13, 84, 85]. A standardized approach to testing genetic modification in combination with novel immunosuppressive agents will ideally bring clarity to the optimal combinations.

6. Conclusions

Currently published preclinical data demonstrate that immunosuppressive therapy, typically incorporating costimulation blockade agents, is required for successful engraftment of porcine tissues, even those with considerable genetic modification [9]. This convergence of experimental therapies in the preclinical setting presents a predicament when considering clinical xenotransplantation trials [31]. It is as yet uncertain whether conventional immunosuppressive agents may be effective enough to facilitate engraftment and maintenance

Table 4: Selected studies using CTLA4-Ig in pig-to-nonhuman primate xenotransplantation.

First author (year)	Donor pig	Recipient NHP	Immunosuppressive regimen	Longest survival (days)
Heart xenotransplantation, heterotopic				
Iwase (2015) [96]	GTKO.CD46.CD55	Baboon	ATG, anti-CD20mAb, abatacept, MMF, CS	23
Iwase (2015) [96]	GTKO.CD46.TBM	Baboon	ATG, belatacept, anti-CD40mAb, tacrolimus, MMF, CS	130
Liver xenotransplantation				
Shah (2017) [24]	GTKO	Baboon	ATG, belatacept, tacrolimus, CVF, CS	25
Islet xenotransplantation				
Cordona (2006) [108]	WT	Rhesus	Anti-CD25mAb, anti-CD154mAb, CTLA4-Ig	>260
Hecht (2009) [113]	Fetal pancreatic fragments	Cynomolgus	Anti-CD25mAb, anti-CD154mAb, FTY720, rapamycin, CTLA4-Ig	380
Thompson (2011) [21]	WT	Rhesus	Anti-CD25mAb, anti-CD40mAb, rapamycin, belatacept	203
Thompson (2011) [20]	GTKO	Rhesus	Anti-CD154mAb, anti-LFA1mAb, MMF, belatacept	249
Thompson (2012) [79]	WT	Rhesus	MMF, belatacept, alefacept, anti-LFA1mAb, tacrolimus	114
Graham (2013) [114]	WT	Cynomolgus	Anti-CD25mAb, abatacept, tacrolimus, rapamycin	>180

NHP: nonhuman primate; WT: wild-type; ATG: anti-thymocyte globulin; CVF: cobra venom factor; MMF: mycophenolate mofetil; mAb: monoclonal antibody; CS: corticosteroids; GTKO: α1,3-galactosyltransferase gene knockout; TBM: thrombomodulin.

of genetically modified ("humanized") porcine organs or tissues. Furthermore, many of the immunosuppressive agents currently being tested in nonhuman primate models are not yet approved for clinical use. More rigorous testing of novel genetically modified pigs with minimal and/or more clinically relevant immunosuppression is warranted. However, the potential of costimulation blockade in xenotransplantation holds great promise for future use. Although genome-edited pig xenografts will certainly minimize the need for novel immunosuppressive agents, the increasing depth of our costimulation blockade library will benefit the future of allotransplantation and xenotransplantation alike.

Abbreviations

APC: Antigen-presenting cells
AHXR: Acute humoral xenograft rejection
Gal: Gal α(1,3) Gal
GTKO: α1,3-Galactosyltransferase gene knockout
LFA: Lymphocyte function-associated antigen
mAb: Monoclonal antibody
MHC: Major histocompatibility complex.

Conflicts of Interest

None of the authors has a conflict of interest.

Authors' Contributions

This manuscript has been revised and approved by all authors.

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